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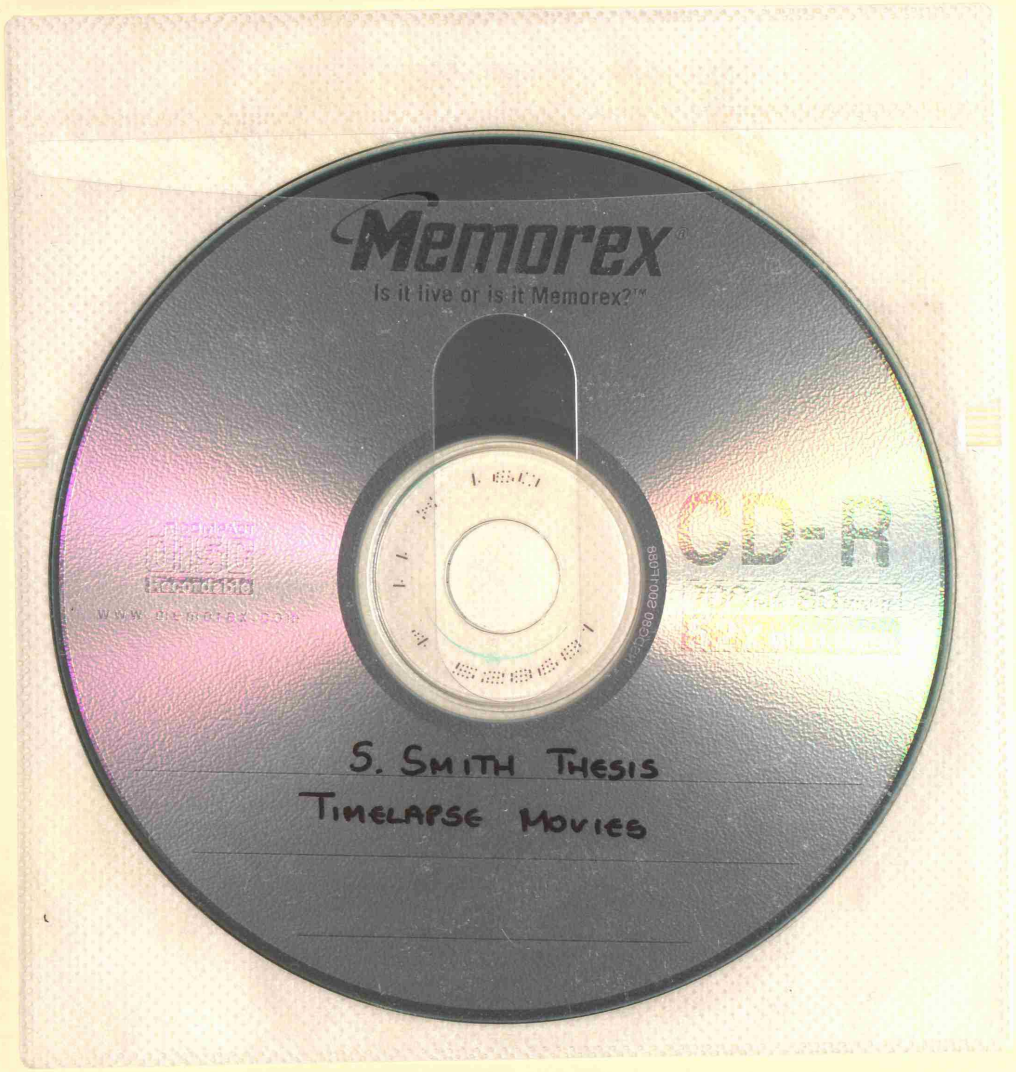

# The regulation and role of PAK1 in macrophages

by

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**Abstract**

The p21-activated kinases (PAKs) are a family of serine/threonine kinases that are activated by the Rho GTPases, Rac1-3, Cdc42, Chp, TC10 and Wrch-1, as well as a number of lipids and PAK-interacting proteins. PAKs have been implicated in a variety of cellular processes including cell polarisation, migration, chemotaxis and gene transcription. The aim of this study was to determine the regulation and function of PAK1, using bone marrow-derived macrophages (BMMs) as a model system.

CSF-1 stimulation of BMMs induced rapid phosphorylation and activation of PAK1, and CSF-1 and TNF$\alpha$ also promoted an increase in PAK1 protein levels after 2 to 5 hours. The rise in PAK1 protein was not due to changes in gene transcription, mRNA translation or reduced proteasomal degradation. Wildtype and PAK1$^{-/-}$ BMMs were compared to determine the roles of PAK1 in a number of macrophage functions. PAK1 was required for maximal ERK, p38 and JNK activation in response to CSF-1 although it did not appear to signal via c-Raf or MEK1. PAK1 phosphorylated Op18 and LIMK downstream of CSF-1, which regulate microtubule and actin reorganisation respectively. PAK1 also regulated MLC phosphorylation although this was not a CSF-1-induced response.

PAK1$^{-/-}$ BMMs adhered more rapidly than WT BMMs and transiently spread to a greater area than WT BMMs. PAK1 promotion of ERK activity at the lamellipodial edge was required for the stability of lamellipodial extension during cell spreading with a greater number of smaller lamellipodia produced in PAK1$^{-/-}$ BMMs compared to WT BMMs. However, PAK2, active Cdc42, total ERK, $\beta$-PIX and Rac1 all localised normally at the cell periphery in spreading PAK1$^{-/-}$ BMMs. PAK1 was also required for membrane ruffling after CSF-1 stimulation but was dispensable for macrophage polarisation, migration and chemotaxis towards CSF-1. PAK1, therefore, contributes to CSF-1 and cell adhesion induced signalling in macrophages.

## Abbreviations

| | |
|---|---|
| ADF | Actin depolymerising factor |
| ADP | Adenosine diphosphate |
| AID | Auto-inhibitory domain |
| ALLnL | N-acetyl leucyl-leucyl norlucinal |
| Arf | ADP ribosylation factor |
| APC | Adenomatous polyposis coli |
| APS | Ammonium persulphate |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| BMM | Bone marrow-derived macrophage |
| CA | Constitutively active |
| CaMK | Calcium-Calmodulin Dependent Protein Kinase |
| cDNA | Complementary deoxyribonucleic acid |
| $CO_2$ | Carbon dioxide |
| CRIB | Cdc42/Rac interactive binding domain |
| CSF-1 | Colony stimulating factor-1 |
| CSF-1R | CSF-1 receptor |
| C-terminal | Carboxyl terminus |
| C3 transferase | *Clostridium botulinum* 3-transferase |
| CXCL1 | CXC motif ligand 1 |
| $ddH_2O$ | Double distilled water |
| Dia | Diaphanous |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethylsulphoxide |
| DN | Dominant negative |
| DNA | Deoxyribonucleic acid |
| DRF | Diaphanous-related formin |
| DTT | Dithiothreitol |
| ECL | Enhanced chemi-luminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| Ena/Vasp | Enabled/vasodilator-stimulated phosphoprotein |

| | |
|---|---|
| ERK | Extracellular signal-regulated kinase |
| Etk/Bmx | Endothelial tyrosine kinase/ Bone marrow X kinase |
| F-actin | Filamentous actin |
| FAK | Focal adhesion kinase |
| FACS | Fluorescent activated cell sorting |
| Fc$\gamma$R | Fc-gamma receptor |
| FCS | Foetal calf serum |
| FITC | Fluorescein isothiocynate |
| FLIM | Fluorescence lifetime imaging microscopy |
| FRET | Fluorescence resonance energy transfer |
| GAP | GTPase activating protein |
| GDP | Guanine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GIT | Grk interactor |
| GPCR | G-protein coupled receptor |
| GST | Glutathione S-transferase |
| GTP | Guanine triphosphate |
| HRP | Horse radish peroxidase |
| IL-1 | Interleukin-1 |
| IPTG | Isopropylthiogalactopyranoside |
| IRES | Internal Ribosome Entry Site |
| IRSp53 | Insulin receptor substrate 53 kDa |
| IQGAP1 | IQ motif containing GTPase activating protein 1 |
| JNK | c-*jun* N-terminal kinase |
| KD | Kinase dead |
| LIMK | Lim domain kinase |
| LPA | lysophosphatidic acid |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocytic chemotactic peptide-1 |
| MEK1 | MAP kinase kinase 1 |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| mRNA | Messenger ribonucleic acid |

| | |
|---|---|
| MT | Microtubule |
| MTOC | Microtubule organising centre |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NET1 | Neuroepithelioma transforming gene 1 |
| NF-$\kappa$B | Nuclear factor-kappa B |
| NP40 | Nonidet P40 |
| N-terminal | Amino terminus |
| N-WASp | Neural-Wiskott-Aldrich syndrome protein |
| Op18 | Oncoprotein 18 |
| PAGE | Polyacrylamide gel electrophoresis |
| PAK | p21-activated kinase |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PDK1 | 3-Phosphoinositide-dependent protein kinase-1 |
| PE | Phycoerythrin |
| PH | Pleckstrin homology |
| PI3-kinase | Phosphoinositide 3-kinase |
| PI(4)P 5-K | Phosphatidylinositol 4-phosphate 5-kinase |
| PIX | PAK-interacting exchange factor |
| PKA | cAMP Dependent Protein Kinase |
| PKC | Protein kinase C |
| PMSF | Phenylmethylsulphonylfluoride |
| PTEN | Phosphatase and tensin homolog |
| Pyk2 | Proline-rich tyrosine kinase 2 |
| RhoGDI | Rho guanine nucleotide dissociation inhibitor |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROCK | Rho kinase |
| RT | Room temperature |
| RTK | Receptor tyrosine kinase |
| RT-PCR | Reverse transcription polymerase chain reaction |

| | |
|---|---|
| S.D | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| S.E.M | Standard error of the mean |
| Ser | Serine |
| Siglec-1 | Sialic acid binding Ig-like lectins-1 |
| SRE | Serum response element |
| SRF | Serum response factor |
| SSH-1L | Slingshot |
| TBE | Tris-borate-EDTA |
| TBS | Tris buffered saline |
| Temed | N,N,N',N'-Tetramethylethylenediamine |
| Thr | Threonine |
| TIRF | Total internal reflection fluorescence |
| TNF$\alpha$ | Tumour necrosis factor alpha |
| Tris | Tris (hydroxymethyl) aminomethane |
| TRITC | Tetramethyl rhodamine iso-thiocyanate |
| Tyr | Tyrosine |
| UV | Ultra violet |
| WASp | Wiskott-Aldrich syndrome protein |
| WAVE | WASP-family verprolin homologous protein |
| $WH_2$ | WASp homology domain 2 |
| WT | Wild type |

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# Chapter 1: Introduction.

## 1.1: The cytoskeleton

A number of different cellular processes such as cell migration and phagocytosis require dynamic changes in cell morphology. Cell shape and the ability to extend or retract membrane in a controlled manner are largely reliant upon the cytoskeleton.

The cytoskeleton consists of three types of protein filaments, actin, microtubules and intermediate filaments (IFs). Each of these filament types plays a specific role in maintaining the shape and structure of the cell and in regulating various cellular processes.

### 1.1.1: Actin

The formation of polarised actin filaments is essential for many cellular functions. Filaments of actin (F-actin) are 7 nm in diameter and formed from double helical polymers of actin subunits arranged in a head to tail formation which gives the filament polarity. The barbed end is generally orientated outwards, towards the cell periphery and is the site of polymerisation, whilst the pointed end is generally orientated inwards and is the main site of disassembly (see review (Pollard and Borisy, 2003)). Formation of actin filaments requires ATP-bound actin monomers and the presence of $Mg^{2+}$, although this is believed to stabilise the monomer rather than be directly linked to polymerisation (De La Cruz et al., 2000a). Instead, ATP hydrolysis and ADP dissociation appear to act as an internal timer that regulates filament disassembly.

A large number of proteins are involved in regulating the formation, disassembly, stability and branching of actin filaments. Profilin is an ATP-actin monomer binding protein that targets actin to the barbed end of filaments (Kang et al., 1999) ensuring polarised growth. Addition of actin monomers to the barbed end is believed to be the driving force behind

membrane extension indicating the importance of this process (see review (Pollard and Borisy, 2003)). Profilin binding to actin monomers is competed for by thymosin-$\beta$4, a protein that sequesters ATP-actin, preventing its addition to nucleating filaments (De La Cruz et al., 2000b). Actin filament extension is further regulated through binding of capping proteins (such as CapZ and gelsolin) which prevent addition of ATP-actin monomers to the barbed end of filaments (Barkalow et al., 1996). Capping of filaments is believed to increase the pool of free ATP-actin monomers by disassembly from their pointed ends, whilst 'funnelling' these free monomers to sites of barbed end growth as specifically required (Carlier and Pantaloni, 1997). Other proteins such as Ena/Vasp compete with capping proteins for the barbed end of actin filaments and bind profilin, promoting actin polymerisation, whilst inhibiting the binding of capping proteins (Barzik et al., 2005).

The generation of new barbed ends has been shown to occur through three mechanisms. The most researched mechanism is stimulation of the Arp2/3 complex by WASp/Scar proteins which promotes branching of new actin filaments off pre-existing filaments (Machesky and Insall, 1998; Mullins et al., 1998). Another key mechanism more recently identified is through the diaphanous-related formins (DRFs) (see review (Faix and Grosse, 2006)). The DRFs consist of three proteins, Dia1, 2 and 3 and have been implicated in the production of filopodia (Schirenbeck et al., 2005) and stress fibres (Hotulainen and Lappalainen, 2006) downstream of the Rho GTPases. Their mechanism of action is still unclear but it is believed they attach to the barbed ends of actin filaments and allow actin nucleation whilst remaining attached to the barbed end (see review (Wallar and Alberts, 2003) and (Harris and Higgs, 2004)). A third mechanism of actin nucleation has also been identified in *Drosophila*. The protein Spire, contains four WASp homology 2 (WH2) domains with each one capable of binding an actin monomer. It is currently thought that Spire binds actin filaments at each WH2 domain and aligns them to promote the formation of an actin filament (see review (Baum and Kunda, 2005) and (Quinlan et al., 2005)).

Conversely, it is also important for old filaments to be severed, allowing the release of actin monomers for re-use. Severing proteins, such as ADF (Actin depolymerising factor)/cofilin bind ADP-actin filaments and promote their disassembly by introducing a twist into the actin helix (McGough et al., 1997). Cofilin is believed to be critical for lamellipodia extension in stimulated cells by producing free barbed ends at the membrane (DesMarais et al., 2005). This allows an increase in the actin filament 'treadmilling' which is the driving force behind cell migration (see review (Paavilainen et al., 2004)).

This tight control of actin filament regulation allows the cell to produce distinct membrane extensions such as lamellipodia which are broad, thin membrane extensions and filopodia, membrane spikes that extend outwards. Both of these structures are formed by forces generated by actin filaments, but the underlying actin architecture is very different. Lamellipodia are formed from a dense meshwork of branched actin filaments behind the cell membrane where a number of barbed ends face the membrane (Resch et al., 2002), presumably to generate enough force to push the membrane forward (Figure 1.1A). Filopodia, however, are formed from parallel bundles of actin filaments, that can then extend and produce the characteristic spike of membrane (Biyasheva et al., 2004) (Figure 1.1B). Formation of filopodia and dorsal stress fibres are believed to be through the DRFs (Hotulainen and Lappalainen, 2006) whilst lamellipodia are believed to utilise Arp2/3 complex-mediated actin nucleation (Machesky and Insall, 1998).

A) B)

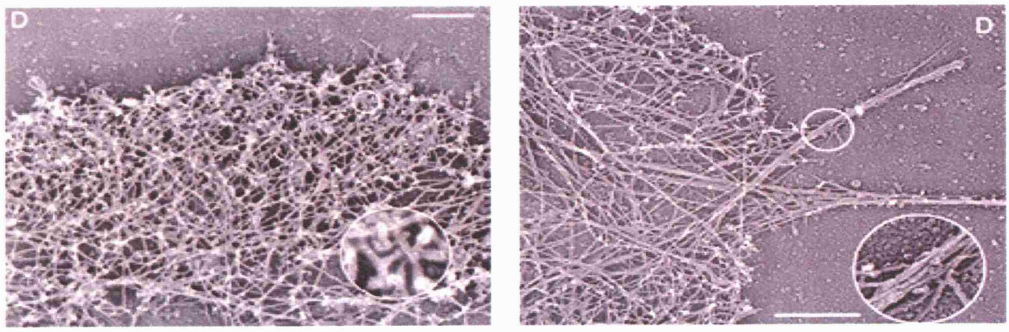



**Figure 1.1:** Electron micrographs (EM) of the actin cytoskeleton
A) EM of a lamellipodium showing the F-actin meshwork. Inset shows close up of branched actin filaments. B) EM showing the tightly bundled actin

filaments forming a filopodium. Inset shows close up of an F-actin bundle. Images taken from (Biyasheva et al., 2004).

### 1.1.2: Myosin

The myosins are a large superfamily of proteins capable of binding F-actin, hydrolyzing ATP and generating force and movement. Myosins typically have three subdomains, the motor domain which interacts with F-actin and ATP, the neck domain which binds light chains or calmodulin and the tail domain, which anchors and positions the motor domain. Myosin II is classed as 'conventional' myosin and is composed of two heavy chains and two pairs of light chains. It is unique in that it can form filaments through self-association via the $\alpha$-helical tail domain (see review (Sellers, 2000)). Myosin II filaments and actin filaments can interact allowing myosin-generated force to be exerted upon the actin cytoskeleton (Svitkina et al., 1997). This locally produced and controlled force can promote changes in cell shape, contraction during motility and in cytokinesis. Myosin II can be regulated by a number of kinases and will be discussed in detail later.

### 1.1.3: Microtubules

Microtubules (MTs) are filaments formed from the parallel polymerisation of GTP-bound tubulin heterodimers into protofilaments. Interactions between the protofilaments produce cylindrical microtubules with a diameter of 24 nm (see review (McKean et al., 2001)). Of the 5 types of tubulin identified, $\alpha$ and $\beta$ tubulin form microtubules (Downing and Nogales, 1999) whilst $\gamma$ tubulin forms a ring complex within the centrosome which is responsible for the nucleation of microtubules (Zheng et al., 1995). $\delta$ tubulin is localised in both the cytoplasm and the nucleus and is enriched at the spindle poles during mitosis (Smrzka et al., 2000) and $\epsilon$ tubulin is localised at the centrosome and is required for the assembly of basal bodies (Dupuis-Williams et al., 2002).

Like actin filaments, MTs are polarised filaments with the minus end of the microtubule usually anchored in the microtubule organising centre (MTOC)

and the plus end growing out towards the cell periphery. The ability of MTs to traverse the cell makes them ideal for intracellular transport. The polarised nature of microtubules allows motor proteins to move along them in a specific direction. Motors such as kinesin I move towards the plus ends of microtubules targeting their cargoes to the cell periphery, promoting membrane polarisation whilst, dynein motor proteins transport cargoes to the minus ends of MTs. Microtubules are, therefore, critical for the transport of various cellular components such as vesicles and organelles (see reviews (Vaughan, 2005; Welte, 2004)).

### 1.1.4: Intermediate filaments

The intermediate filaments (IFs) are 10 nm filaments formed *in vitro* in the absence of ATP and GTP and for many years were considered little more than static structural networks. However, more recent data has shown them to be dynamic members of the cytoskeletal network of proteins. Five families of IFs, consisting of over 50 proteins in humans (see review (Fuchs and Cleveland, 1998)), have been identified with four cell type specific cytoplasmic families and one ubiquitous nuclear family (see review (Helfand et al., 2004)).

The intermediate filaments are targets of intracellular signalling. Vimentin is targeted by a number of kinases, including PAK (p21-activated kinase) (Goto et al., 2002) and PKC$\epsilon$ (Protein kinase C). Phosphorylation of IFs is required for their remodelling in processes such as cytokinesis (Kawajiri et al., 2003) and phosphorylation of vimentin by PKC$\epsilon$ was shown to be critical for recycling of $\beta$1 integrin vesicles back to the membrane and for cell migration (Ivaska et al., 2005). This supports data from vimentin$^{-/-}$ fibroblasts which have migratory defects (Eckes et al., 1998). Vimentin may also influence cell adhesion due to its localisation at podosomes (Correia et al., 1999) as well as its role in integrin recycling.

### 1.2: The Rho GTPases

The Rho GTPases are members of the Ras superfamily of monomeric GTP-binding proteins and were shown to act as 'molecular switches'. Twenty two members of the family have been identified in humans but the most-studied members are RhoA, Rac1 and Cdc42 (Figure 1.2).

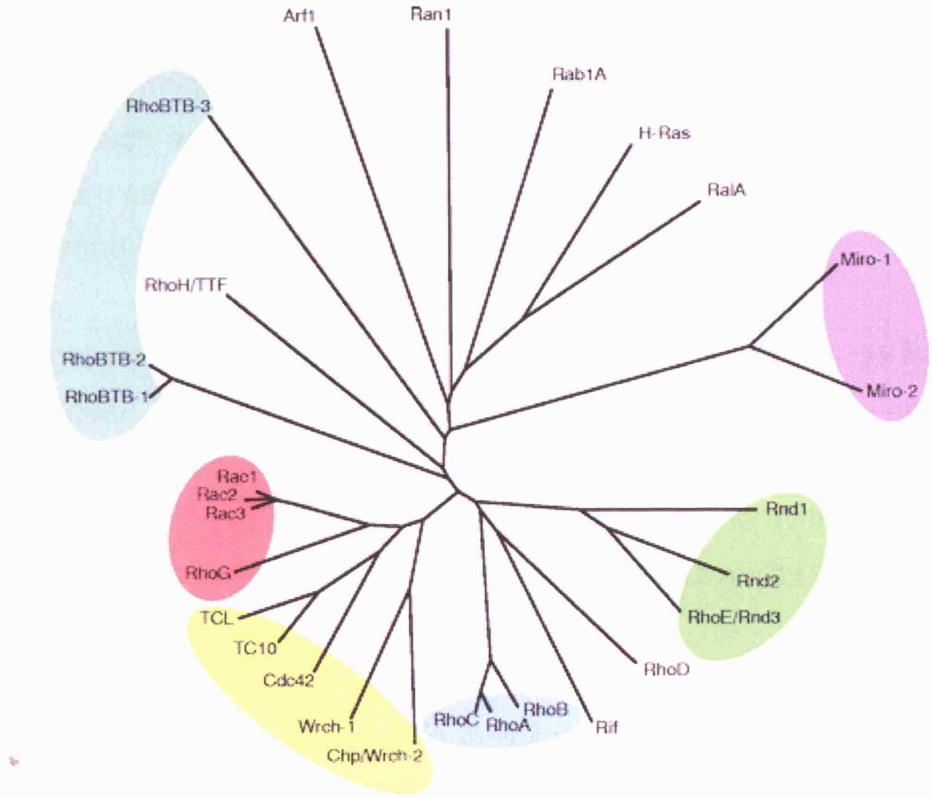



**Figure 1.2:** Phylogenetic tree of Rho GTPases and representatives of other Ras-superfamily GTPases. Analysis shows the Rho GTPases can be divided into six major groups: RhoA related, Rac-related, Cdc42-related, Rnd-related, RhoBTB proteins and Miro proteins. Taken from (Wennerberg and Der, 2004).

#### 1.2.1: Rho GTPase regulation

Most Rho GTPases exist in two forms, an inactive GDP-bound form and an active GTP-bound form. Cycling between these two forms allows regulation of their downstream signalling pathways (Figure 1.3) although the Rnd

proteins and RhoH are likely to be GTPase deficient and not regulated through this cycling (see review (Wennerberg and Der, 2004)).

Activation is promoted by guanine nucleotide exchange factors (GEFs), proteins that facilitate the exchange of GDP for GTP on the Rho protein. Over 60 GEFs have been identified in the human genome indicating the high level of regulation that occurs on these pathways (see review (Rossman et al., 2005)). The Rho GTPases localise to the cell membrane through carboxyl (C)-terminal prenylation and can bind to effector proteins involved in processes such as cytoskeletal reorganisation, cell cycle progression, gene transcription, cell migration and cell adhesion (see reviews (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002b)).

Down regulation of Rho signalling is induced through another large family of proteins (over 70 members identified in humans) called GTPase-activating proteins (GAPs). Coupling of GAPs to active Rho proteins enhances the intrinsic rate of GTP hydrolysis by the Rho GTPases. The hydrolysis of GTP results in a conformational change in Rho, reducing its ability to interact with its effector proteins (Ihara et al., 1998).

Rho guanine nucleotide dissociation inhibitors (RhoGDIs) also regulate Rho GTPase activity. Three mammalian RhoGDIs have been identified which sequester Rho GTPases in the cytoplasm through binding and masking the Rho prenylation moiety at their C-termini. The amino (N)-terminus of RhoGDIs then inhibits spontaneous GDP-GTP exchange (Gosser et al., 1997; Longenecker et al., 1999) preventing Rho GTPase targeting to the membrane and activation. However, not all Rho GTPases bind RhoGDIs, such as Chp and TC10 (see review (DerMardirossian and Bokoch, 2005)).

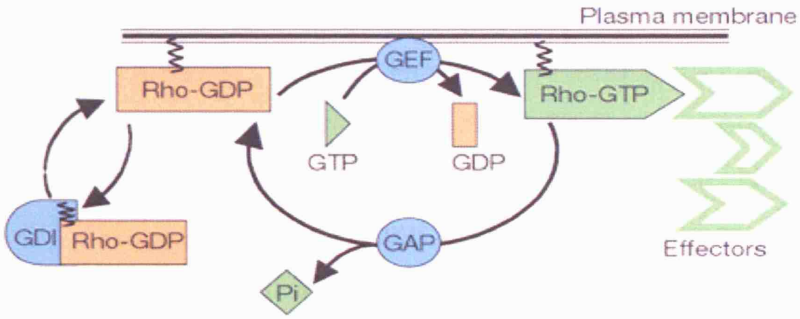



**Figure 1.3:** Regulation of Rho GTPase activity
Inactive Rho GTPases are activated via GEF proteins which promote GDP release allowing GTP to bind and activation. GAPs facilitate Rho GTPase downregulation by enhancing the hydrolysis of GTP. RhoGDIs bind Rho GTPases and sequester them in the cytoplasm, preventing activation. Diagram taken from (Etienne-Manneville and Hall, 2002a).

A number of bacterial cytotoxins have been identified that are capable of regulating Rho GTPase activity. The cytotoxins are frequently utilised within the laboratory, with the most frequently used being the C3 exoenzyme, a toxin expressed by *Clostridium botulinium.* C3 has been shown to irreversibly ADP-ribosylate RhoA, RhoB and RhoC, inhibiting their function and making it a useful tool in the study of Rho function. Other toxins inhibit RhoA, Rac and Cdc42 through glucosylation (Toxin A and B), proteolysis (YopT) and through mimicking Rho GAPs (YopE) (see review (Aktories and Barbieri, 2005)).

### 1.2.2: Rho GTPase signalling

The Rho GTPases have been implicated in a number of signalling pathways affecting various aspects of cellular function.

#### 1.2.2.1: Rho GTPase transcriptional effects

As well as regulating the cytoskeleton for processes such as cell migration, the Rho GTPases have been implicated in regulation of gene transcription. RhoA, Rac1 and Cdc42 were shown to activate serum response factor (SRF), a transcription factor that binds serum response elements (SRE) (Hill

et al., 1995) and generally regulates cell growth and differentiation (see review (Chai and Tarnawski, 2002)). RhoA, Rac1 and Cdc42 also efficiently induce signalling by the transcription factor NF-κB (Hayden and Ghosh, 2004), although they were not required for UV-induced NF-κB activity (Perona et al., 1997). RhoA, Rac1 and Cc42 are capable of activating the p38 mitogen-activated protein kinase (p38 MAPK) pathway and/or the c-*jun* N-terminal kinase (JNK) (Coso et al., 1995; Minden et al., 1995) in certain cell types. Whilst CNK1 binding promotes RhoA-induced activation of JNK (Jaffe et al., 2005), Minden *et al.* propose that PAK is the downstream effector that links Rac1 and Cdc42 signalling to p38 MAPK and JNK activation. Indeed, PAK has been implicated in these pathways and will be discussed in detail later (Chapter 1.3.6).

Cdc42 may also have a role in regulating translation. The nuclear cap-binding complex (CBC) is a heterodimer that targets the 7-methylguanosine cap on RNA. Growth factor stimulation of cells was shown to enhance CBC targeting to the RNA cap which appeared to involve Cdc42 signalling (Wilson et al., 1999). Signalling to the CBC is critical for the splicing of precursor mRNAs and appears to be Cdc42- and S6 kinase-dependent (Wilson et al., 2000). Rac1 may also influence mRNA translation. Rac1 was shown to regulate integrin-mediated upregulation of Cyclin D1 mRNA translation via PI3-K, Shc and FAK (Mettouchi et al., 2001). This suggests that Cdc42 and Rac1 may also have a role in RNA processing as well as gene transcription.

#### 1.2.2.2: Rho GTPase effects on the cell cycle

Microinjection of CA RhoA, Rac1 or Cdc42 into quiescent fibroblasts promoted progression of the cell cycle through the $G_1$ checkpoint and induced DNA synthesis. This appeared to be via the JNK pathway rather than the ERK pathway that is activated by the Ras GTPase signalling pathway (Olson et al., 1995). RhoA has also been implicated in the regulation of cytokinesis through its regulation of actomyosin contractility (see review (Piekny et al., 2005)), whilst Cdc42, via mDia3, is required for microtubule capture at kinetochores during mitosis (Yasuda et al., 2004). The

Rho GTPases may, therefore, contribute both to $G_1$/S phase transition and cell division.

### 1.2.2.3: Signalling to the actin cytoskeleton

Early research implicated the Rho GTPases as regulators of the actin cytoskeleton. Microinjection studies indicated that RhoA was responsible for the formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992), Rac1 for the formation of membrane ruffles and lamellipodia (Ridley et al., 1992) and Cdc42 stimulates the extension of filopodia (Nobes and Hall, 1995).

A number of downstream targets have been identified for RhoA, Rac1 and Cdc42 (see review (Bishop and Hall, 2000) and Figure 1.4). The principle RhoA effector is ROCK (Rho-kinase) of which two isoforms have been identified. ROCK influences the actin cytoskeleton through phosphorylation of LIMK, to promote inhibition of cofilin and actin filament stability (Maekawa et al., 1999). ROCK also enhances actomyosin contractility through phosphorylation of myosin light chain phosphatase (MLCP) (Kawano et al., 1999) and myosin light chain (MLC) directly (Amano et al., 1996). Another key Rho target is the DRF family protein; Dia1. RhoA activation of Dia1 promotes its binding to F-actin barbed ends and the actin-binding protein profilin (Watanabe et al., 1999). Dia1 promotes actin polymerisation and nucleation whilst remaining bound to the actin barbed end, moving in a processive manner in the presence or absence of profilin. However, the presence of profilin enhances filament elongation speed (Kovar et al., 2006).

The key downstream effectors of Rac1 include PAK, IRSp53 and IQGAP all of which can regulate the actin cytoskeleton. Rac1 binding to IRSp53 is believed to activate WAVE (Miki et al., 2000) promoting the formation of membrane ruffles through the Arp2/3 complex (Suetsugu et al., 1999) although recent data suggests that IRSp53 optimises the activity of a WAVE2-Sra1-Nap1-Abi1-HSPC300 signalling complex at the cell membrane (Suetsugu et al., 2006). Activation of this complex by Rac1 promotes Arp2/3

complex actin nucleation. Another target is IQGAP which can bind both Rac and Cdc42. Stimulation of IQGAP was shown to have a diverse range of cellular effects but has been implicated in binding actin in lamellipodia and stimulation of filopodia through stabilisation of Cdc42 activity (Briggs and Sacks, 2003). Rac1 was also implicated in the activation of phosphatidylinositol 4-phosphate 5-kinase (PI(4)P 5-K) increasing the production of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$). Capping protein binds PtdIns(4,5)$P_2$ removing it from the end of actin filaments allowing fresh actin polymerisation at the cell membrane (Tolias et al., 2000). However, Rac activation of PI(4)P 5-K is controversial and further study is required. Rac1 regulation of PAK will be discussed in detail later (Chapter 1.3.3).

Cdc42 also regulates the actin cytoskeleton upon activation. Cdc42 is known to target PAK, as Rac1 does, suggesting potential crosstalk between the pathways, again, this will be discussed in detail later (Chapter 1.3.3). Another mechanism whereby Cdc42 regulates the actin cytoskeleton is through activating WASp/N-WASp. The haematopoietic cell-specific Wiskott-Aldrich syndrome protein (WASp) and its ubiquitously expressed relative neural-WASp (N-WASp) induce actin polymerisation (Miki et al., 1996; Symons et al., 1996) and link Cdc42 signalling to the Arp2/3 complex (Rohatgi et al., 1999) although other methods of regulation may also be critical (Cory et al., 2002). However, Cdc42's role in this process may not be universal with some research indicating Cdc42 is not essential for directional migration, filopodium formation or polarisation in fibroblastoid cells (Czuchra et al., 2005). It is possible that other Rho GTPases are also utilised. The GTPase Rif was recently shown to induce filopodium formation via Dia2 (Pellegrin and Mellor, 2005) indicating potential overlap in Rho GTPase function although different mechanisms are utilised. Another Cdc42 target implicated in actin regulation is IRSp53. Previously regarded as a Rac1 target, Cdc42 interaction with IRSp53 was shown to induce Mena, a member of the Ena/Vasp family, to induce filopodium formation (Krugmann et al., 2001). This suggests that Rac1 and Cdc42 may both target a number of the same effectors but produce different actin-based structures. For instance, where

Rac1 promotes IRSp53-WAVE2-mediated lamellipodia extension, Cdc42 reduces the IRSp53-WAVE2 interaction (Suetsugu et al., 2006). This may be possible as different binding sites were implicated for Rac1 and Cdc42 in IRSp53 (Krugmann et al., 2001; Miki et al., 2000) although it is also possible that different IRSp53 splice variants were used by the two groups.

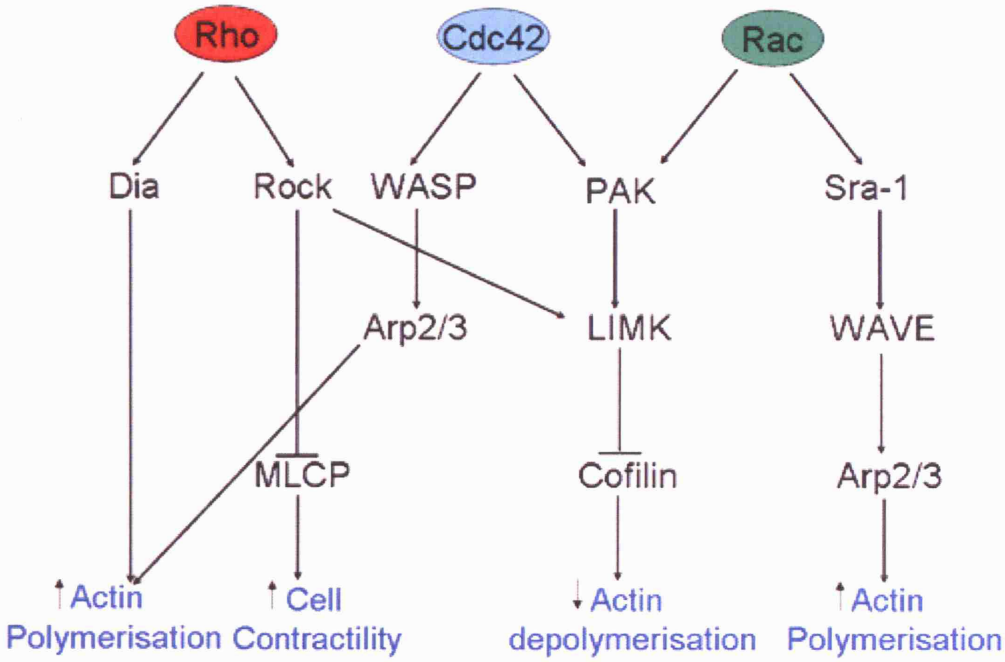



**Figure 1.4:** Diagram of the key Rho GTPase signalling targets involved in regulation of the actin cytoskeleton.

### 1.2.2.4: Signalling to microtubules

As well as regulating the actin cytoskeleton, RhoA, Rac1 and Cdc42 have all been implicated in regulation of microtubules. RhoA activation of the formin Dia2 increased microtubule stability as identified through levels of detyrosinated tubulin (Cook et al., 1998; Palazzo et al., 2001) whilst Rac1 can signal through PAK (Daub et al., 2001; Wittmann et al., 2004) and IQGAP1 (Fukata et al., 2002) to increase microtubule stability and capture respectively. Cdc42 is capable of signalling through PAK and IQGAP1, but can also promote MTOC polarisation through a pathway involving Par6, an aPKC, glycogen synthase kinase-3$\beta$ (GSK-3 $\beta$) and APC (Adenomatous Polyposis Coli) which captures microtubules at the leading edge (Cau and

Hall, 2005; Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003; Ohno, 2001). The Rho GTPases therefore frequently regulate both the actin and microtubule cytoskeleton through the same downstream targets suggesting crosstalk between the pathways.

### 1.2.2.5: Rho GTPases in cell migration

#### i) Lamellipodium formation

The effects of Rho GTPases on the cytoskeleton suggest they are likely to be key regulators of cell migration (Figure 1.5). The ability of Rac1 to regulate WAVE activity via an IRSp53/Abi1 complex leads to the formation of membrane ruffles and lamellipodia through the Arp2/3 complex (Kheir et al., 2005; Miki et al., 2000; Suetsugu et al., 2006; Suetsugu et al., 1999). Rac1 inhibition was initially shown to prevent cell migration (Allen et al., 1998; Nobes and Hall, 1999) but more recent research has indicated that Rac1-null BMMs have defects in cell spreading and ruffling but not in migration (Wells et al., 2004; Wheeler et al., 2006b). The earlier research used dominant negative (DN) constructs which have been shown to sequester GEFs and could explain the discrepancy seen between the studies. Another possibility is that haematopoietic specific Rac2 may replace Rac1 in macrophage migration as it is implicated in neutrophil chemotaxis (Carstanjen et al., 2005). In BMMs, $\alpha_4\beta_1$ integrin-induced migration was shown to be dependent upon Syk-Rac2 signalling (Pradip et al., 2003) although Rac1-null neutrophils exhibited impaired chemotaxis (Glogauer et al., 2003). These data indicate that Rac signalling is vital for cell migration. However, other members of the Ras GTPase superfamily have also been implicated in lamellipodium formation. A CA Rab5 mutant, a Rab GTPase implicated in endocytosis was shown to induce lamellipodium formation and migration independently of Rac (Spaargaren and Bos, 1999). RhoA can also induce lamellipodium formation in colon cancer cells plated onto laminin (O'Connor et al., 2000) whilst RhoG, possibly through regulation of Rac1 (Katoh et al., 2006) and Cdc42 both induce lamellipodium formation in expression studies (Aspenstrom et al., 2004). This suggests that lamellipodium formation is probably regulated by

factors such as the extracellular matrix, cell stimuli and cell type and that a single Rho GTPases does not completely regulate the process. Rac ability to inhibit cofilin activity via PAK and LIMK may also regulate lamellipodium formation as regulation of cofilin activity is important for lamellipodium extension and migration (Chen et al., 2001) and active cofilin at the leading edge of a cell is vital for maintaining cellular polarity (Dawe et al., 2003). It is possible that Rac-mediated inhibition of cofilin maintains a leading edge lamellipodia through prevention of competing lamellipodia in other areas of the cell.

**ii) Adhesion formation and disassembly**

Another important aspect of cell migration is the formation and turnover of cell-substrate adhesions. Two main types of adhesions exist, focal adhesions and focal contacts. Focal complexes are generally transient integrin-mediated adhesions to the extracellular matrix and are regarded as the initial stages of adhesion. As they mature, focal complexes develop into focal adhesions, larger, more stable integrin-associated adhesions which are tethered to actin stress fibres and provide firmer adhesion for the cell (see review (Carragher and Frame, 2004)). The key regulators of cell adhesion are the integrins, heterodimeric receptors that bind the extracellular matrix as well as induce intracellular signalling pathways in conjunction with a family of cell surface proteoglycans called syndecans (Beauvais and Rapraeger, 2003). Activated integrins are localised to the leading edge of migrating cells and new adhesions are formed in a Rac-dependent manner (Kiosses et al., 2001). Integrin activation has been shown to induce Rac and Cdc42 activation (Price et al., 1998) suggesting a potential feedback loop where active Rac recruits integrins to the leading edge inducing integrin activation and further Rac activation. One downstream effector of Rac thought to be involved in adhesion formation and turnover is PAK. Integrin adhesion was shown to regulate Rac activation of PAK (del Pozo et al., 2000). This will be discussed in more detail later (Chapter 1.3.5), but PAK appears to be required for both formation and disassembly of focal complexes/adhesions (Brown et al., 2002; Kiosses et al., 1999). A number of other proteins are

also involved in adhesion turnover including focal adhesion kinase (FAK), Src and extracellular signal-regulated kinase (ERK). FAK is regarded as being critical for the dynamic regulation of integrin-based adhesions in migrating cells, whilst Src is also localised with FAK within the adhesion where it promotes signalling through phosphorylation of a number of targets including FAK and paxillin (see review (Jones et al., 2000) and (Thomas et al., 1999)). ERK, meanwhile, is involved in extension but not retraction of pseudopodia in non-motile cells (Brahmbhatt and Klemke, 2003) and targets to newly formed focal adhesions upon integrin engagement (Fincham et al., 2000). Localisation of ERK to focal adhesions may target downstream effectors, such as paxillin, to regulate adhesion and migration through changes to FAK and Rac activity (Ishibe et al., 2004).

**iii) Cell body contraction and tail retraction**

For cells to migrate persistently, it is necessary for them to pull the rear of the cell forward. Use of the Rho inhibitor C3-transferase and DN RhoA mutants implicated RhoA in regulation of cell contractility. Macrophages microinjected with C3-transferase showed an elongated phenotype suggesting loss of contractility (Allen et al., 1997). RhoA is believed to exert this effect through its effector ROCK. Activation of ROCK by RhoA can regulate contractility through MLC phosphatase and direct phosphorylation of MLC (Amano et al., 1996; Kawano et al., 1999). Phosphorylation of these targets promotes actomyosin contractility and resultant cell contraction.

Rac and Cdc42 are likely to influence contractility through PAK which acts as an antagonist for ROCK-mediated phosphorylation of MLC. PAK was shown to phosphorylate and inhibit MLCK decreasing actomyosin contractility (Sanders et al., 1999) although this was contradicted in endothelial cells as CA PAK1 increased phosphorylated-MLC levels (Kiosses et al., 1999). Regulation of contractility by these pathways is likely to be controlled through compartmentalisation. ROCK may regulate MLC phosphorylation within the cell body whilst MLCK regulates MLC at the cell periphery (Totsukawa et al., 2004; Totsukawa et al., 2000). Cdc42 can also influence contractility through

the myotronic dystrophy kinase-related Cdc42-binding kinase (MRCK) which can phosphorylate and inhibit the MLC phosphatase resulting in increased MLC phosphorylation (Wilkinson et al., 2005).

Retraction of the cell tail during migration may be the limiting step for migration speed in cells with a reasonable level of adhesion. Calpain-mediated cleavage of adhesion proteins is reportedly required for disassembly of adhesions at the rear of the cell (Palecek et al., 1998). It is possible that stretch-activated calcium channels are activated in spreading cells and the influx of $Ca^{2+}$ ions could activate calpain leading to the cleavage of adhesions (Lee et al., 1999). A role of ERK in this process may also be present because of its ability to activate calpain at the cell membrane (Glading et al., 2001).

Other research, however, suggests that microtubule-dependent delivery of 'relaxing' signals promotes adhesion disassembly (Kaverina et al., 1999). This disassembly appears to involve MLC. Loss of myosin II or PAKa from *Dictyostelium* cells led to impaired tail retraction (Chung et al., 2001) whilst inhibition of myosin II through blocking Rho or ROCK signalling produced a similar phenotype in monocytes and neutrophils (Worthylake and Burridge, 2003; Xu et al., 2003).

**iv) Chemotaxis**

Chemotaxis is the directed migration of a cell up a chemoattractant gradient. A role in environmental sensing for filopodia was first observed in neuronal growth cones (Davenport et al., 1993) and Cdc42's ability to promote filopodium formation (Kozma et al., 1995) led to the discovery that Cdc42 is required for directional sensing in cell types other than neurons (Allen et al., 1998; Nobes and Hall, 1999). This role of Cdc42 is presumably in the formation of bundled actin filaments required for filopodia projection. Cdc42 promotes this process through interactions with WASp/N-WASp and its downstream target the Arp2/3 complex (Castellano et al., 1999; Rohatgi et al., 1999). However, despite filopodia being vital for neuronal directional

migration, their requirement in other cell types is unclear. It is possible that Cdc42's involvement in chemotaxis is through activating the Par6/aPKC complex inducing microtubule and cell polarity (Cau and Hall, 2005; Etienne-Manneville and Hall, 2001).

Regulation of cofilin phosphorylation through LIMK and its phosphatase slingshot (SSH-1L) may also be important for directional cell migration. The correct spatial and temporal regulation of cofilin activity was critical to allow both lamellipodium formation at the leading edge but also to prevent unnecessary membrane extension around other parts of the cell. If incorrectly regulated cells are incapable of chemotaxing due to the loss of a single dominant lamellipodium (Nishita et al., 2005). LIMK is a downstream target of PAK1 and ROCK whereas SSH-1L has been shown to be activated upon binding to F-actin but interestingly is reported to be inhibited by PAK4 (Soosairajah et al., 2005). Macrophages do not express PAK4 (Figure 3.1), suggesting that either this regulation is lost in macrophages or that other PAKs are also capable of inhibiting SSH-1L.

Other factors also contribute to directed migration. One pathway that appears to be critical in some cells is the generation of phosphatidylinositol (3, 4, 5)$P_3$ by PI3-K (see review (Merlot and Firtel, 2003)). The importance of PI3-K was highlighted in PI3-K$\gamma$-null neutrophils which exhibited reduced chemotaxis due to a reduction in leading edge persistence and enhanced membrane extensions around the surface of the cell (Hirsch et al., 2000). Use of isoform-specific antibodies and inhibitors confirmed that PI3-K$\beta$ and PI3-K$\delta$ also have a role in chemotaxis (Sadhu et al., 2003; Vanhaesebroeck et al., 1999) although the effect each PI3-K has upon chemotaxis appears to be cell type or stimulus-specific. T-lymphocytes were shown to use PI3-K$\gamma$ whilst B-cells use PI3-K$\delta$ for chemotaxis (Reif et al., 2004).

The role of PI3-K in chemotaxis is to provide localised production of PtdIns(3, 4, 5)$P_3$ at the leading edge allowing the targeting of a number of effector proteins. Effector proteins that contain the necessary PH domain to localise to these sites include Rho GEFs such as Vav and $\alpha$-PIX (PAK interacting exchange factor) (Han et al., 1998; Yoshii et al., 1999) which promote the

activation of Rac and Cdc42. The presence of α-PIX at the leading edge also implicates PAK in regulation of chemotaxis. PAK can be targeted to the leading edge via α-PIX and activated there by Rac and Cdc42. This will be discussed in further detail later (Chapter 1.3.5).

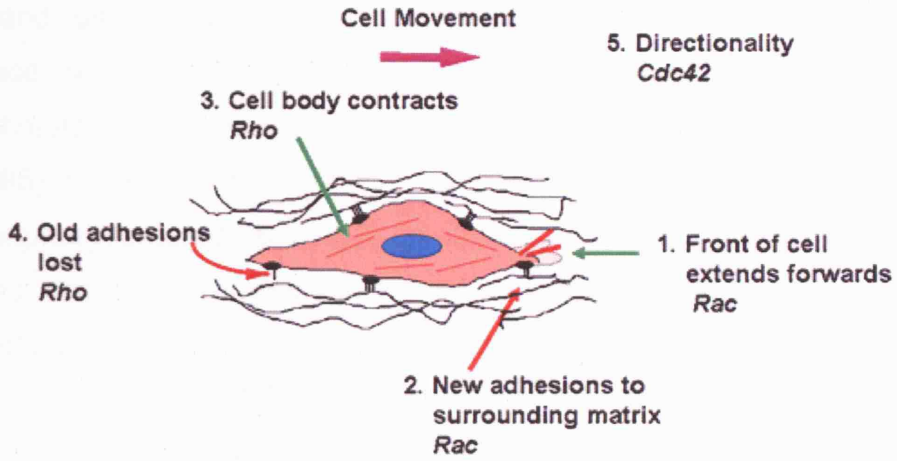



**Figure 1.5:** Regulation of cell migration. Taken from (Ridley et al., 2003).

### 1.2.2.6: Rho GTPases and cell spreading

The ability of suspended cells to attach and spread is vital, particularly for leukocytes which must interact and spread on the endothelium to allow passage out of the vasculature into the surrounding tissues. Monocytes in the bloodstream are required to undergo extravasation at sites of infection. Initially monocytes 'roll' along the endothelium through a loose attachment by the cell surface adhesion proteins, selectins (Ivetic et al., 2004). Endothelial cells in the region of an infection present chemoattractant molecules (e.g. CSF-1, MCP-1) on their cell surface (Lu et al., 1998; Pixley and Stanley, 2004) to promote firmer, integrin-based adhesions (Hogg et al., 2002) allowing slight spreading and migration across the endothelium to locate a cell junction for diapedesis.

Total internal reflection fluorescence (TIRF) microscopy of mouse embryonic fibroblasts adhering to fibronectin allowed spreading to be modelled mathematically (Dobereiner et al., 2004). Analysis showed that lamellipodia contract periodically during spreading depending on the matrix rigidity, fibronectin binding and MLCK activity. These contractions generate rearward

waves of F-actin that are believed to stimulate the next phase of membrane extension (Giannone et al., 2004).

Regulation of MLCK also appears to be critical for spreading (Giannone et al., 2004). MLCK regulates actomyosin contractility via phosphorylation of MLC and use of a fluorescently tagged MLCK construct indicated its presence at the lamellipodial leading edge (Giannone et al., 2004). Phosphorylated MLC was localised at the rear of lamellipodia (Matsumura et al., 1998) suggesting a mechanism whereby MLCK activation of MLC at the cell periphery is essential for cell spreading. However, MLC phosphorylation was reduced upon adhesion to a rigid substratum (Wakatsuki et al., 2003) suggesting the initial induction of spreading does not require actomyosin tension. The Rho GTPases may regulate this mechanism. PAK1, downstream of either Rac1 or Cdc42 is capable of inhibiting MLCK (Sanders et al., 1999) and MLCP (Takizawa et al., 2002), whilst ROCK downstream of RhoA can also inhibit MLCP (Kimura et al., 1996). This indicates actomyosin contractility is tightly regulated during spreading.

Integrin engagement induces Rho GTPase activity (see review (DeMali et al., 2003)) and Rac and Cdc42 activation by integrins is essential for fibroblast spreading (Price et al., 1998). Rac1 was identified as a critical small GTPase involved in spreading through its ability to promote lamellipodium extension (Choma et al., 2004; Vidali et al., 2006; Wells et al., 2004) but a number of other upstream and downstream targets have also been identified. Proteins identified as upstream activators of Rac1 in spreading include Cdc42 (Price et al., 1998), Vav2 (Marignani and Carpenter, 2001), Vav1 although in a GEF-independent manner (del Pozo et al., 2003), Rap1 (Arthur et al., 2004), $\mu$-Calpain (Kulkarni et al., 2002; Kulkarni et al., 1999), PAK2 (Obermeier et al., 1998) and PAK1 (Ten Klooster et al., 2006). Downstream of Rac1, FAK and Src are implicated in spreading (Brown et al., 2005) as is WAVE1 (Yamazaki et al., 2005) and Rac1 regulation of myosin II heavy chain (MHC) (van Leeuwen et al., 1999).

Cell spreading also involves the formation and turnover of new adhesions. Rho GTPases can increase cellular levels of intracellular adhesion molecule-

1 (ICAM-1) and E-selectin in leukocytes under specific stimuli (see review (Cernuda-Morollon and Ridley, 2006)). Importantly, however, the Rho GTPases have also been implicated in the formation and turnover of adhesions (Kiosses et al., 2001). Rac has been implicated in the formation of focal contacts within lamellipodia (Allen et al., 1997; Nobes and Hall, 1995; Rottner et al., 1999) but may also affect adhesion disassembly via signalling through PAK (Zhao et al., 2000b) and via antagonism of RhoA signalling (Sander et al., 1999).

### 1.2.2.7: Rho GTPases and immune responses

As well as their fundamental effects as signal transducers, Rho GTPases also have specific roles within cells of the immune system including regulation of leukocyte motility, phagocytosis and production of radical oxygen species (ROS).

#### i) Formation of the phagocytic cup

Phagocytosis is the process whereby foreign particles and apoptotic cells are engulfed and internalised by cells such as macrophages which are 'professional' phagocytes and are an important line of defence against infection. Other cell types are also professional phagocytes including neutrophils and eosinophils.

Different cell surface receptors involved in phagocytosis use different mechanisms to stimulate internalisation. The Fc$\gamma$ Receptors (Fc$\gamma$Rs) bind to micro-organisms that have been opsonised with the antibody IgG, whilst the complement receptor 3 (CR3) binds to targets that have been coated with complement 3bi (C3bi). Fc$\gamma$R-mediated phagocytosis involves the formation of membrane protrusions and ruffling to engulf the particle, whereas CR3-mediated phagocytosis appears to result in the bound particle sinking into the macrophage rather than being engulfed (see review (Castellano et al., 2001)).

Investigations into the differences in signalling during these phagocytic events showed that Fc$\gamma$R-mediated phagocytosis utilised Rac1 and Cdc42 whilst CR3-mediated phagocytosis used integrin-mediated activation of Rho (Caron and Hall, 1998; Wiedemann et al., 2006). However, recent data have implicated Rac and Rho in both mechanisms of phagocytosis, although Rho is not involved in the polymerisation of F-actin at the site of phagocytosis (Hall et al., 2006). Phagocytosis of apoptotic cells, meanwhile, requires Rac1, Cdc42 and PI3-K$\beta$ but not RhoA signalling (Leverrier et al., 2003; Leverrier and Ridley, 2001).

To determine the localisation and timing of Rho GTPase activity during Fc$\gamma$R-mediated phagocytosis, fluorescence resonance energy transfer (FRET) microscopy was used. Cdc42 was activated only at the leading edge of the cell, whilst Rac1 was broadly activated throughout the phagocytic cup in an Fc$\gamma$R cytoplasmic domain- and Src-dependent manner. Rac1 and the GEF Vav also co-localised during this process (Cougoule et al., 2006). During closure of the phagosome, Rac1 and Rac2 activity both increased in the phagosomal membrane whilst Cdc42 activity remained localised to the cell periphery (Figure 1.6) (Hoppe and Swanson, 2004).

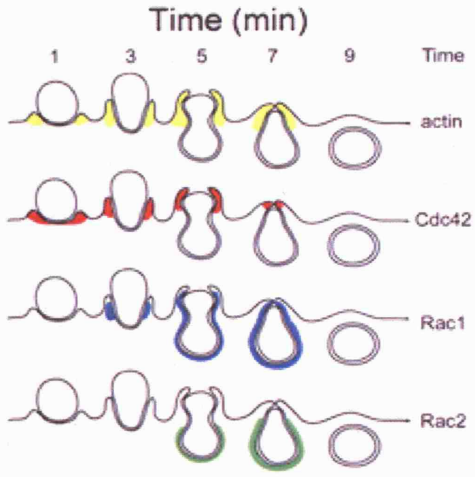



**Figure 1.6:** Localisation of Rho GTPase activity during Fc$\gamma$R mediated phagocytosis. Taken from (Hoppe and Swanson, 2004).

**ii) Production of radical oxygen species**

An important response to phagocytosis in phagocytes is the production of superoxide and radical oxygen species (ROS) via NADPH oxidase. This multicomponent enzyme produces superoxide anion, which rapidly dismutates to hydrogen peroxide and other ROS which, at least in neutrophils, are pumped into the phagocytic vacuole inducing a charge across the membrane. The movement of compensating ions produces conditions in the vacuole which promote microbial killing and digestion by enzymes released into the vacuole from granules in the cytoplasm (see review (Segal, 2005)).

The Rho GTPases Rac1 and Rac2 have long been implicated in the production of ROS (Abo et al., 1991; Knaus et al., 1991). However, as Rac2 is the predominant isoform in neutrophils, it is generally believed to be the key Rho GTPase in the pathway. $Rac2^{-/-}$ macrophages and neutrophils have defective superoxide production and phagocytosis (Li et al., 2002; Yamauchi et al., 2004) indicating its importance in this pathway. However, the stimulus in this process also appears to be important as superoxide production upon serum-opsonised zymosan was normal in $Rac2^{-/-}$ macrophages (Yamauchi et al., 2004). Specificity of Rac isoforms in this process is conferred through a short polybasic domain in the C-terminus which associates with the membrane. Expression of Rac2 in $Rac2^{-/-}$ BMMs rescued the NADPH oxidase defects whilst Rac1 did not. However, exchange of the polybasic region in Rac2 with that of Rac1 prevented rescue (Yamauchi et al., 2005). Cdc42 has also been implicated in the regulation of NADPH oxidase; but, as an antagonist to Rac. Cdc42 acts as a competitive inhibitor and competes with Rac2 for binding to the flavocytochrome b unit of NADPH oxidase (Diebold et al., 2004). This prevents Rac binding and results in the inhibition of ROS production.

## 1.3: The p21-activated kinase family

The p21-activated kinases (PAKs) are an evolutionarily conserved family of serine/threonine kinases. In mammals, six family members have been identified and PAK orthologues have been identified in both budding and fission yeast, slime moulds, worms and flies (see review (Hofmann et al., 2004) and Figure 1.7). The PAKs in higher eukaryotes are divided into two groups based on their homology. All PAKs contain an N-terminal Cdc42/Rac Interaction Binding Domain/p21 Binding Domain (CRIB/PBD) and a C-terminal protein kinase domain, however, the Group A PAKs (PAKs 1-3) also have a number of N-terminal proline-rich domains that allow binding to SH3 domains in other proteins. Differences also exist in PAK activation between groups. The Group A PAKs are capable of binding Cdc42 and Rac1 which disrupts an auto-inhibitory conformation leading to their activation. The Group B PAKs (PAK 4-6), however, preferentially bind Cdc42 although Rac1 can bind, but do not appear to be activated upon binding suggesting Cdc42 interaction determines localisation rather than activity (see review (Jaffer and Chernoff, 2002)).

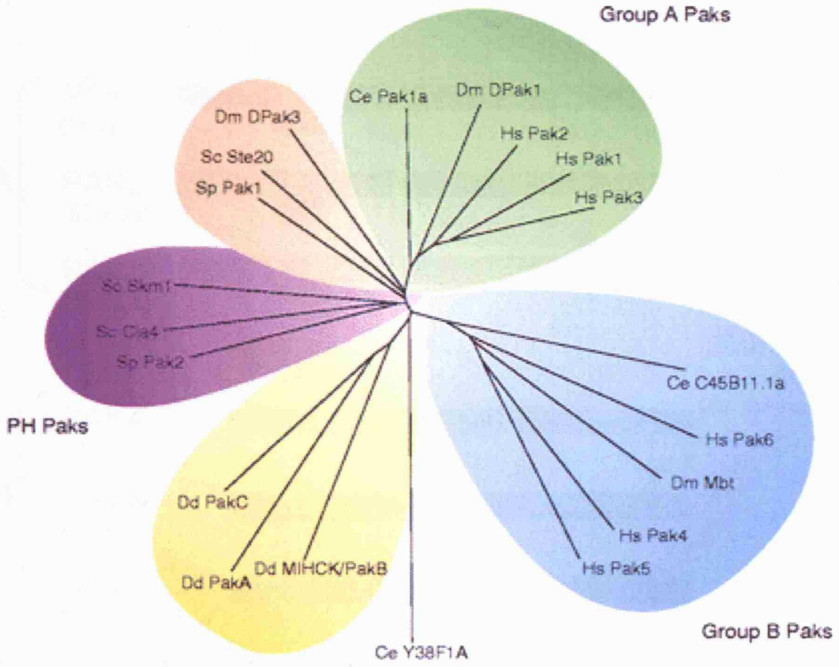



**Figure 1.7:** Phylogenetic tree of PAKs.
In higher eukaryotes, the PAKs make up two separate groups, A (Green) and B (Blue). The budding and fission yeast have a related family of PAKs that contain an N-terminal PH domain (Purple) but also other PAKs without this (Orange). PAKs from slime mould form a separate group (Yellow). Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*. Taken from (Hofmann et al., 2004)

In mammalian PAKs there is reasonable homology between the group members. Homology between Group A PAKs is 88% although within the kinase domain it is 93%. The Group B PAKs share approximately 60% homology in the N-terminal region and 75% within the kinase domain; although homology between the groups kinase domains is much lower (Jaffer and Chernoff, 2002; Pandey et al., 2002). The differences in homology between the groups are evident from diagrams of their basic structures. Whilst the Group A PAKs have a number of N-terminal proline-rich regions, the Group B PAKs just have the CRIB domain (Figure 1.8).

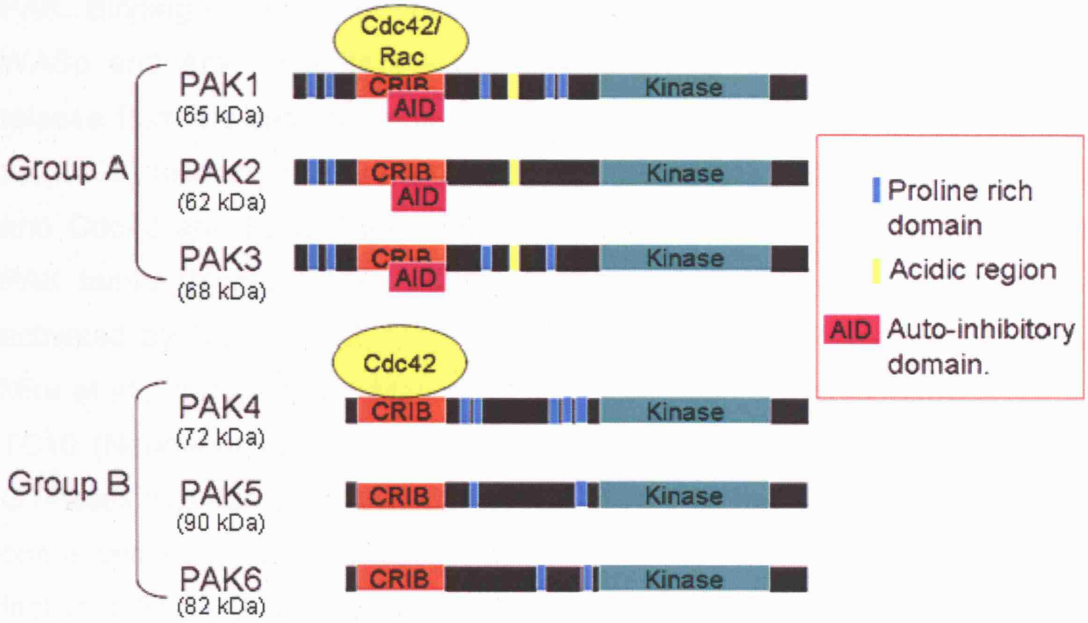



**Figure 1.8:** The basic structure of the six mammalian PAKs.
Diagram showing the basic structures of the Group A and B PAKs from mammals. Adapted from (Jaffer and Chernoff, 2002).

As well as differing structurally, the PAKs have also been shown to have different expression profiles (Table 1.1).

| | |
|---|---|
| PAK1 | Brain, Muscle, Spleen |
| PAK2 | Ubiquitous |
| PAK3 | Brain |
| PAK4 | Colon, Prostate, Testis |
| PAK5 | Brain |
| PAK6 | Prostate, Testis |

**Table 1.1:** Expression profile of the mammalian PAKs (Kumar and Vadlamudi, 2002).

### 1.3.1: PAK regulation

PAK activity is generally regarded to be controlled through Rac and Cdc42 binding to the CRIB domain located in the N-terminus of PAK (Thompson et al., 1998). Inactive PAK exists in an auto-inhibitory dimer with the auto-inhibition domain (AID) of one PAK binding to the kinase domain of another

PAK. Binding of active Rac or Cdc42 to the CRIB domain (also present in WASp and Ack) disrupts the folded structure of the AID resulting in its release from the catalytic region of the dimer partner kinase domain (Lei et al., 2000) relieving auto-inhibition of PAK (Lei et al., 2000). Although Rac1 and Cdc42 are the GTPases commonly regarded as the activators of the PAK family (Manser et al., 1994), PAK1 has been shown to bind and be activated by Rac1, Rac2, Rac3, (Knaus et al., 1998; Manser et al., 1994; Mira et al., 2000) Cdc42 (Manser et al., 1994), Chp (Aronheim et al., 1998), TC10 (Neudauer et al., 1998) and Wrch-1 (Tao et al., 2001). All the Rho GTPases that can bind PAK are closely related suggesting an evolutionarily conserved sequence responsible for the binding. This is supported by the fact that RhoA-G and other Ras superfamily members are not capable of binding PAK. It has been suggested that GTPase binding to PAK is insufficient for full activation (Buchwald et al., 2001; Chong et al., 2001; Zenke et al., 1999). It was reported that phosphorylation is required for full activation and it is possible that binding of Rac or Cdc42 regulates PAK localisation to the membrane where a number of kinases reside.

Upon disassembly of the PAK dimer, PAK1 is autophosphorylated at Thr423 (Zenke et al., 1999) (Thr402 in PAK2 (Gatti et al., 1999; Yu et al., 1998)), a site located within the PAK kinase activation loop. Phosphorylation of this site is believed to inhibit a return to auto-inhibition but also fully activates the kinase domain (Zenke et al., 1999). However, evidence suggests that, *in vivo*, PDK1 is responsible for phosphorylation of PAK1 at Thr423 (King et al., 2000). Phosphorylation also occurs at other sites within PAK that are believed to influence kinase activity and maintenance of activity. One site is Ser144, which lies within the AID and is autophosphorylated upon activation to promote PAK activity (Chong et al., 2001). Another phosphorylation site within PAK1 is Ser21; Akt phosphorylation of this site is involved in the regulation of Nck binding to PAK1 and potentially regulation of cell migration (Zhou et al., 2003). PAK1 is therefore capable of autophosphorylating to regulate its activity and is also targeted by other kinases to alter its localisation and binding partners.

It is interesting to note that the Group B PAKs do not become appreciably activated upon GTPase binding (Abo et al., 1998; Kaur et al., 2005; Lee et al., 2002). The Group B PAKs do not have an identifiable AID like the Group A PAKs although one group have reported the identification of an AID and evidence of Cdc42 activation in PAK5 (Ching et al., 2003) and truncated forms of PAK4 and PAK6 lacking the N-terminus both exhibit enhanced activity. This indicates the presence of some form of intramolecular regulation of activity (Abo et al., 1998; Yang et al., 2001).

As well as GTPase activation, a number of other activation mechanisms have been identified (Figure 1.9).

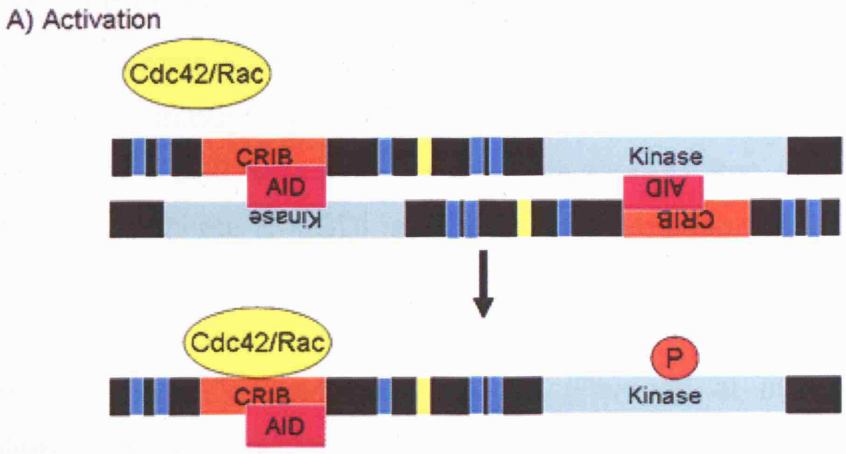
**1. Cleavage** – The PAKs were first identified as kinases that could autophosphorylate and activate upon limited protein digestion (Benner et al., 1995). Caspase 3 digestion of PAK2 has been identified as part of an apoptosis response, producing a catalytically active fragment (Lee et al., 1997; Rudel and Bokoch, 1997).

**2. Lipids** – It was reported that PAK interaction with certain lipids at the membrane is capable of overcoming PAK auto-inhibition. PAK1 can be activated by sphingosine and sphingosine-derived lipids as well as gangliosides and phosphatidic acids with their site of action the same or similar as the GTPases (Bokoch et al., 1998).

**3. Protein interaction** – The GTPases Rac and Cdc42 can bind to PAK promoting its activation. However, other proteins have also been identified that appear to be capable of this. These include $\alpha$- and $\beta$-PIX which can bind PAK and activate PAK independently of Rac/Cdc42 and its GEF activity (Daniels et al., 1999; Feng et al., 2002). PAK is also capable of binding the adapter protein Nck (Zhao et al., 2000a) which is believed to regulate PAK targeting to cell surface receptors (Li et al., 2001) as is the adapter protein Grb2 which localises to growth factor receptors (Puto et al., 2003). Also implicated in PAK activation upon binding are Filamin A and the G$\beta\gamma$ subunit. PAK interaction with Filamin A promoted PAK activity which in turn activated

Filamin A resulting in cytoskeletal reorganisation and membrane ruffling (Vadlamudi et al., 2002) whilst PAK interaction with G$\beta\gamma$ promoted PAK binding to $\alpha$-PIX and Cdc42 activation for chemotaxis (Li et al., 2003). G$\beta\gamma$ is also reportedly capable of activating PAK1 via PI3-K and Akt, independently of Rac and Cdc42 (Menard and Mattingly, 2004).

**4. Phosphorylation** – As described above, PAK is regulated by phosphorylation. The non-receptor tyrosine kinase Etk/Bmx was reported to bind, phosphorylate and activate PAK1 (Bagheri-Yarmand et al., 2001) Another interaction has been reported between PAK2 and the tyrosine kinase Abl. Binding was shown to lead to tyrosine phosphorylation of PAK2, decreasing its activity, providing evidence of a kinase that can inhibit PAK signalling (Roig et al., 2000). PAK1 is also phosphorylated during mitosis by Cdc2 at Thr212 implicating PAK1 in cell cycle control (Thiel et al., 2002). Interestingly, ERK has also been reported to phosphorylate this site (Sundberg-Smith et al., 2005). PAK is downregulated by the phosphatases, partner of Pix (POPX) 1 and 2. POPX1 and 2 are phosphatases capable of interacting with various PIX isoforms and dephosphorylating PAK (Koh et al., 2002) although little is known about the exact mechanism involved.



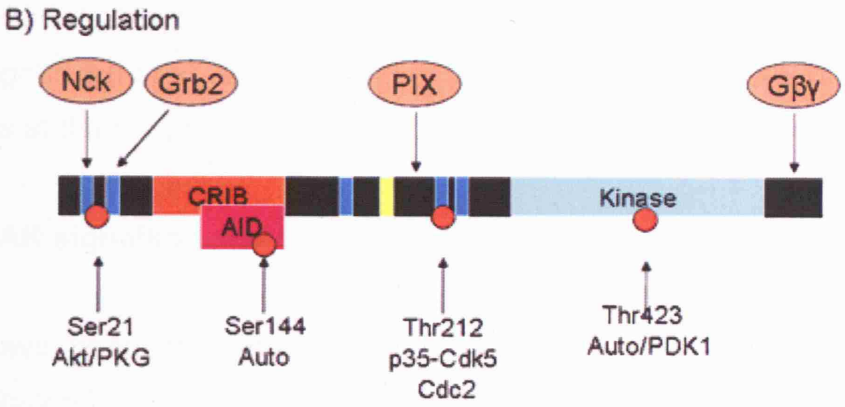



**Figure 1.9:** The activation and regulation of PAK.
Diagram showing (A) Cdc42/Rac-mediated activation of PAK through disruption of a PAK auto-inhibitory dimer and (B), regulation of PAK activity through protein interactions and phosphorylation. Red circles indicate identified sites of phosphorylation with the residue stated that of PAK1.

### 1.3.2: PAK localisation

The localisation of PAK1 in cells indicates a potential role in actin dynamics downstream of the Rho GTPases. Inactive PAK1 is localised in the cytoplasm but is moved to sites of cellular protrusion and focal complexes upon activation by Rac and Cdc42 (see review (Parrini et al., 2005)). Meanwhile, FRET (fluorescence resonance energy transfer) microscopy showed that integrins regulated Rac, and hence PAK, activation at specific membrane sites through release of RhoGDI from Rac (Del Pozo et al., 2002). PAK1 may be responsible for this activation of Rac as it has been shown to phosphorylate RhoGDI leading to Rac's release (DerMardirossian et al., 2004). Other data using FLIM (fluorescence lifetime imaging microscopy) directly showed activation of PAK1 at the cell periphery and in areas of protrusion downstream of Cdc42 (Parsons et al., 2005). The localisation of PAK1, therefore, indicates a likely role in membrane protrusion downstream of Rac1 and Cdc42.

Fewer details exist for the localisation of other PAKs. Caspase-cleaved PAK2 translocates from the cytoplasm to the nucleus during apoptosis (Jakobi, 2004) whilst PAK5 localises to the mitochondria to inhibit apoptosis

(Cotteret et al., 2003). PAK4s ability to interact with growth factor receptors and integrins (Lu et al., 2003; Zhang et al., 2002) meanwhile, suggests PAK4 functions at the periphery of the cell.

### 1.3.3: PAK signalling and targets

PAKs have been implicated in a number of cell signalling pathways and cellular processes (Figure 1.10). Macrophages have been found to express PAK1, 2 and 3 (Figure 3.1) and therefore the signalling pathways activated by the Group A PAKs will be focused upon.

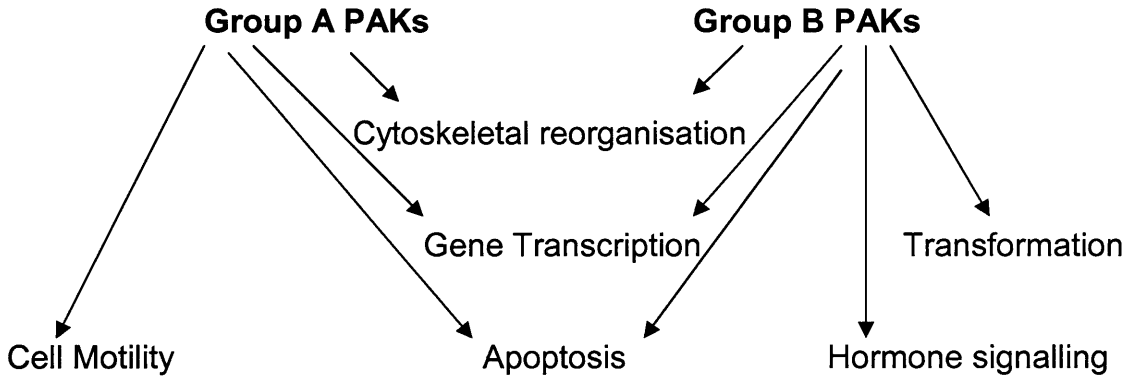



**Figure 1.10:** Cellular processes involving PAK signalling. Adapted from (Jaffer and Chernoff, 2002).

#### 1.3.3.1: PAK regulation of the actin cytoskeleton

As a downstream effector of the Rho GTPases, a number of groups have investigated the role PAK plays in regulation of the actin cytoskeleton.

Early work to determine the function of PAK involved the transfection of cell lines with Rho GTPase and PAK mutants. Use of Rac mutants that do not bind PAK suggested PAK was not required for Rac-induced cytoskeletal reorganisation but was needed for JNK activation (Lamarche et al., 1996). However, transfection of CA PAK1 into Swiss 3T3 cells was shown to induce filopodia, membrane ruffles, dissolution of stress fibres, focal adhesion turnover and retraction of the tail edge (Sells et al., 1997). This suggests a

role for PAK1 in actin dynamics and appears to promote a number of Rac and Cdc42 specific responses, such as the formation of filopodia and membrane ruffles. The contradictory data is possibly a result of cell-type specific responses.

A number of reports have implicated PAK targets as effectors for the regulation of actin dynamics. A major target of PAK is LIM kinase, a two-member family (LIMK-1 and LIMK-2) of serine kinases. PAK1 phosphorylation of LIMK1 is believed to promote LIMK activation, resulting in phosphorylation and inactivation of the actin depolymerising factor, cofilin (Edwards et al., 1999). LIMK can also be regulated by ROCK downstream of Rho (Ohashi et al., 2000) and MRCK$\alpha$ downstream of Cdc42 (Sumi et al., 2001) indicating the critical function it has in actin dynamics. Dephosphorylation and inhibition of LIMK1 is achieved through a family of protein phosphatases called Slingshot (Soosairajah et al., 2005), the same phosphatases that dephosphorylate and activate cofilin (Niwa et al., 2002). The importance of tightly regulating cofilin activity is evident as spatial and temporal regulation of this pathway was critical for directional cell migration of Jurkat T-cells (Nishita et al., 2005) and fibroblasts (Dawe et al., 2003). Further regulation of this pathway was shown as an active PAK4 construct could phosphorylate and inhibit Slingshot (Soosairajah et al., 2005). It had previously been reported that PAK4, as well as PAK1, could bind and phosphorylate LIMK1 (Dan et al., 2001) suggesting that PAK4 is critical in regulating the active levels of LIMK1, Slingshot and hence, cofilin. However, BMMs do not express PAK4 (Figure 3.1).

Another potential mechanism PAK can regulate actin dynamics through is regulation of phosphorylated MLC levels (Sells et al., 1999) and actomyosin contractility (Figure 1.11). PAK1 can phosphorylate and inhibit MLCK decreasing phosphorylated MLC levels (Sanders et al., 1999) and actomyosin contractility. However, PAK1 has also been implicated in the inhibition of Myosin Light Chain Phosphatase (MLCP), a target more commonly associated with ROCK (Totsukawa et al., 2000). PAK1 phosphorylates CPI17 and Myosin Binding Subunit of Type 1 Protein

Phosphatase (MBS), with MBS being a part of the MLCP holoenzyme and CPI17 a specific inhibitor of MLCP activity (Takizawa et al., 2002). The PAK family appear to play an important role in MLC regulation because PAK2 is also capable of phosphorylating MLCK (Goeckeler et al., 2000) and MLC directly (Chew et al., 1998). Therefore, the PAKs are capable of both directly and indirectly promoting MLC phosphorylation but are also capable of inhibiting MLC phosphorylation. This contradictory data is probably a result of cell/system differences. Whilst the PAK1 and PAK2 phosphorylation of MLCK was observed in HeLa cells, PAK2 phosphorylation of MLC was detected in bovine pulmonary artery endothelial (BPAE) cells and the PAK1 regulation of MLCP was performed *in vitro*.

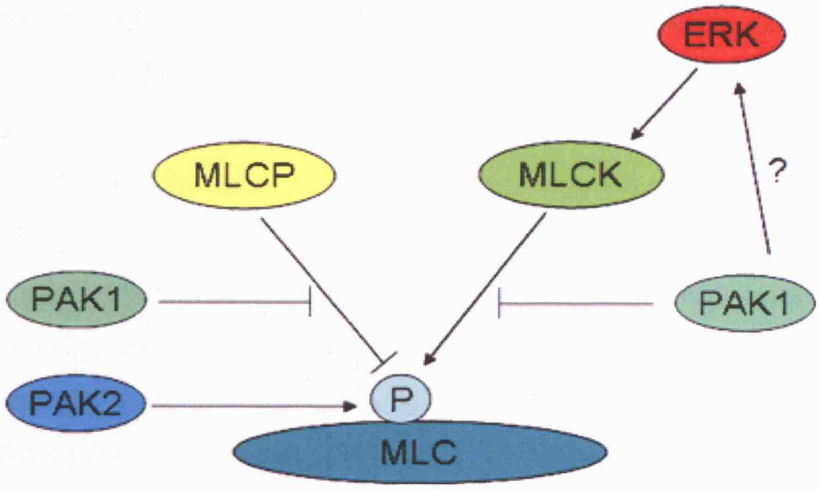



**Figure 1.11:** PAK mediated regulation of MLC phosphorylation
Diagram showing PAK regulation of MLC phosphorylation through inhibition of MLCK and MLCP. PAK1 may also promote ERK-mediated activation of MLCK, whilst PAK2 is capable of phosphorylating MLC directly.

As stated in Chapter 1.3.1, PAK1 is capable of being activated by and activating Filamin A. Filamin A functions as an actin cross-linking protein binding actin filaments within the cell to give the network more rigidity and strength and promoting filament branching (see review (Stossel et al., 2001)). However, it also appears to affect actin dynamics as PAK1 requires Filamin A for formation of dorsal membrane ruffles (Vadlamudi et al., 2002) which are absent after stimulation in Filamin A-deficient cells (see review (Stossel et al., 2001)). This suggests that membrane ruffling downstream of

PAK, which is reported to be a Rac-dependent pathway (Wells et al., 2004) may signal through Filamin A, although a number of other pathways are also likely to be used. These include PAK activation of β-PIX and p38 (Lee et al., 2001) and Rac activation of WAVE via the Abi1 complex (Kheir et al., 2005; Suetsugu et al., 2006).

Finally, another PAK target, merlin, is implicated in the regulation of actin dynamics. The merlin gene, NF2, is a tumour-suppressor gene and is involved in the formation of neurofibromatosis type II, a cancer characterised by the formation of tumours of the nervous system. Merlin has a high homology to the ezrin-radixin-moesin (ERM) family which link membrane proteins to the cytoskeleton and also localise to areas of membrane remodelling where merlin may regulate actin filament stability (James et al., 2001). Regulation of merlin activity is important due to its proposed role in modulating cell motility and proliferation (see review (Xiao et al., 2003)). Studies showed that CA Rac and Cdc42 induced PAK1-mediated phosphorylation of merlin at Ser518, a site important for its activity and localisation (Xiao et al., 2002). Interestingly, it was also reported that Rac, PAK and merlin exist in a negative feedback loop as merlin is capable of inhibiting PAK1 (Hirokawa et al., 2004).

#### 1.3.3.2: PAK regulation of microtubules

As has been stated (Chapter 1.1.3), microtubules are essential for the intracellular transport of organelles and vesicles allowing the generation of polarised cells. Regulation of microtubule stability is therefore critical for cell polarisation and resultantly cell migration.

Only one target has been identified for PAK1 that regulates microtubules, Oncoprotein 18 (Op18/Stathmin) (Daub et al., 2001; Wittmann et al., 2004). Op18 is a microtubule-destabilising protein although the mechanism by which Op18 promotes microtubule destabilisation is still debated (see review (Cassimeris, 2002)). PAK1 phosphorylation of Op18 at Ser16, however, is believed to inactivate its microtubule destabilising ability (Daub et al., 2001).

### 1.3.4: PAK and cell migration

The Rho GTPases have a well documented role in cell migration (Chapter 1.2.2.4). The ability of PAKs to regulate cytoskeletal reorganisation downstream of Rac and Cdc42 through LIMK, MLCK (Chapter 1.3.3.1) and Op18 (Chapter 1.3.3.2) suggested it may have critical functions in regulating motility (Figure 1.12).

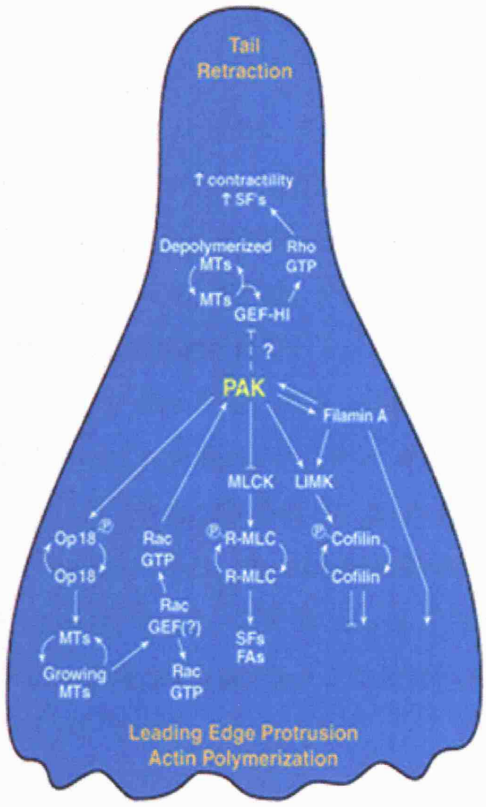



**Figure 1.12:** Hypothetical model of motility regulation by PAK. Taken from (Bokoch, 2003).

A number of studies have reported a role for PAK in cell migration. PAK1 modulation of binding to the adapter protein Nck by Akt phosphorylation was shown to affect cell migration (Zhou et al., 2003) and is consistent with data suggesting PI3-K regulates PAK1-induced migration upon heregulin stimulation (Adam et al., 1998). PAK1 is also implicated in regulation of endothelial cell migration and for the formation and turnover of adhesions within the lamellipodium rather than lamellipodium extension itself (Kiosses et al., 1999). Interestingly, adhesion has been shown to stimulate neuronal

migration through the L1 (CD171) adhesion molecule which stimulates PAK1 via Vav2 (Schmid et al., 2004).

Use of a CA PAK1 construct also implicated PAK1 in migration as squamous cell carcinoma (SCC) cell migration was enhanced (Zhou and Kramer, 2005). Further evidence for a role of PAK1 in migration comes from data suggesting nicotine (Fang and Richardson, 2005) and nischarin (Alahari et al., 2004) can inhibit cell migration through PAK1 inhibition.

PAK is also involved in chemotaxis, the process of directed migration. Chemotaxis towards the chemokines CXCL1 (Wang et al., 2002) and C5a (Li et al., 2003) was shown to require PAK1, possibly in a p38 MAPK-dependent manner (Rousseau et al., 2006) whilst PAK2 was activated during BMM chemotaxis towards the chemokine RANTES (Weiss-Haljiti et al., 2004). Use of a KD PAK1 construct or the PAK AID, which is capable of binding the group A PAK CRIB domain resulting in their inhibition, also inhibited LPA-induced chemotaxis (Jung et al., 2004). A potential mechanism for this is PAK1 regulation of myosin II-B phosphorylation which is essential for chemotaxis (Even-Faitelson et al., 2005) whilst PAK binding to $\beta$-PIX was essential for leukocyte chemotaxis across a restrictive barrier (Volinsky et al., 2006).

PAK1 regulation of cytoskeletal reorganisation (Chapter 1.3.3) and adhesion (Chapter 1.3.5) is therefore likely to be required for the migration and chemotaxis of cells.

### 1.3.5: PAK and cell spreading

The small GTPase Rac1 regulates cell spreading (Wells et al., 2004) making its downstream target PAK1 a likely candidate to also play a role. PAK1 has been implicated in cell spreading after Rac1 activation in platelets (Suzuki-Inoue et al., 2001) and may regulate platelet spreading through regulation of cortactin (Vidal et al., 2002). Loss of VASP was also shown to produce an increase in cell spreading through increased activation of Rac1 and PAK1

(Garcia Arguinzonis et al., 2002). However, the role of PAK1 in regulating Rac1-induced spreading may not be as a simple effector. PAK1 may compete with Rac1 for binding to β-PIX, which allows Rac1 dissociation from RhoGDI, activation and induction of lamellipodium extension (Ten Klooster et al., 2006) (Figure 1.13). Ten Klooster *et al.* also reported a spreading defect and enhanced Rac1 activity in PAK1-null fibroblasts.

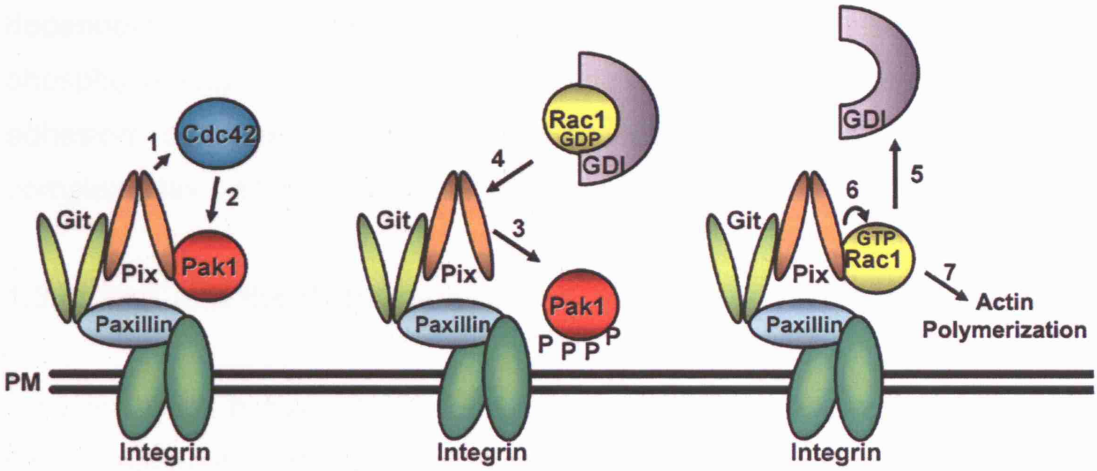



**Figure 1.13:** Proposed model of PAK1 regulation of Rac1-induced spreading. Taken from (Ten Klooster et al., 2006).

The importance of interactions between PAK1 and the PIX family is highlighted by the number of processes the complex is involved in. These include focal adhesion turnover (see review (Rosenberger and Kutsche, 2005)), podosome formation (Webb et al., 2005), regulation of centrosome maturation (Zhao et al., 2005) and promoting Rac1 activation downstream of Cdc42 (Baird et al., 2005). PAK1 may also directly affect cell adhesion through activation of the integrin $\alpha_M\beta_2$ (Jones et al., 1998) and through activation of focal adhesion kinase (FAK). Along with Src, FAK signalling is involved in spreading (Brown et al., 2005), downstream of lysophosphatidic acid (LPA) in human melanoma cells (Jung et al., 2004).

Another possible method through which PAK1 can regulate cell spreading is through its ability to regulate ERK activation (Chapter 1.3.6, Figure 4.2 and (Frost et al., 1996)). ERK localises at the cell membrane (Glading et al., 2001) and in focal adhesions (Kermorgant et al., 2004) and was vital for EGF-mediated fibroblast migration (Glading et al., 2000) through

phosphorylation of m-calpain (Glading et al., 2004). ERK was also shown to be activated upon adhesion in a PAK-dependent manner (Eblen et al., 2002; Sundberg-Smith et al., 2005) and to phosphorylate a site in PAK1 that regulates the cell cycle and post-mitotic spreading in fibroblasts (Thiel et al., 2002). ERK is also reportedly, responsible for paxillin phosphorylation that promotes lamellipodium formation and spreading in a FAK- and Rac-dependent manner (Ishibe et al., 2004). PAK1 is also capable of phosphorylating paxillin at Ser273 which is reportedly responsible for adhesion complex regulation through localisation of a PAK-PIX-GIT1 complex (Nayal et al., 2006).

### 1.3.6: PAK and the MAPK pathways

A potential link between PAK, ERK and spreading is also implicated through PAKs regulation of the MAPK pathways. A role for the PAK family in MAPK regulation initially came from research in yeast. The yeast PAK homologue, Ste20 was found to be a MAPK kinase kinase kinase (MEKK kinase) in the yeast $\alpha$-mating factor signalling pathway (see review (Herskowitz, 1995)) One of earliest pathways identified downstream of PAK was the p38 MAPK pathway (Zhang et al., 1995). Shortly afterwards, it was determined that PAK1 was capable of activating the other MAPK pathways, ERK (Frost et al., 1996) and JNK (Bagrodia et al., 1995; Brown et al., 1996) implicating PAK1 as a key regulator of their activity although activation of the pathways does appear to be cell type specific .

A number of mechanisms have been identified through which PAK can activate the MAPK pathways (Figure 1.14). These include phosphorylation of the MAPK kinases, MEK1 (Pullikuth et al., 2005), MKK3 and MKK6 (Lee et al., 2001) and through phosphorylation of the Ras effector c-Raf which also targets MEK1 (Beeser et al., 2005). However, PAK1 also appears capable of inhibiting JNK through phosphorylation of MEKK1 (Gallagher et al., 2002).

PAK1 was also reported to regulate MAPK activation through regulating the formation of protein complexes. PAK1 can promote MEK1-ERK binding upon

adhesion through phosphorylation of MEK1, which resulted in increased ERK activity. ERK then inhibits PAK1-mediated phosphorylation of MEK1, thereby negatively regulating its own activity (Eblen et al., 2004). Adhesion was also shown to induce a direct interaction between PAK1 and ERK2 which led to PAK1 phosphorylation in an ERK-dependent manner. These data suggest PAK1 acts as a scaffold for MAPK signalling upon adhesion (Sundberg-Smith et al., 2005). The MEK partner 1 (MP1) protein can also bind PAK1 directly and regulate PAK1's phosphorylation of MEK1 upon adhesion. MP1 and its partner, p14, together with MEK1 may also regulate RhoA/ROCK activity, a process necessary for cell spreading (Pullikuth et al., 2005).

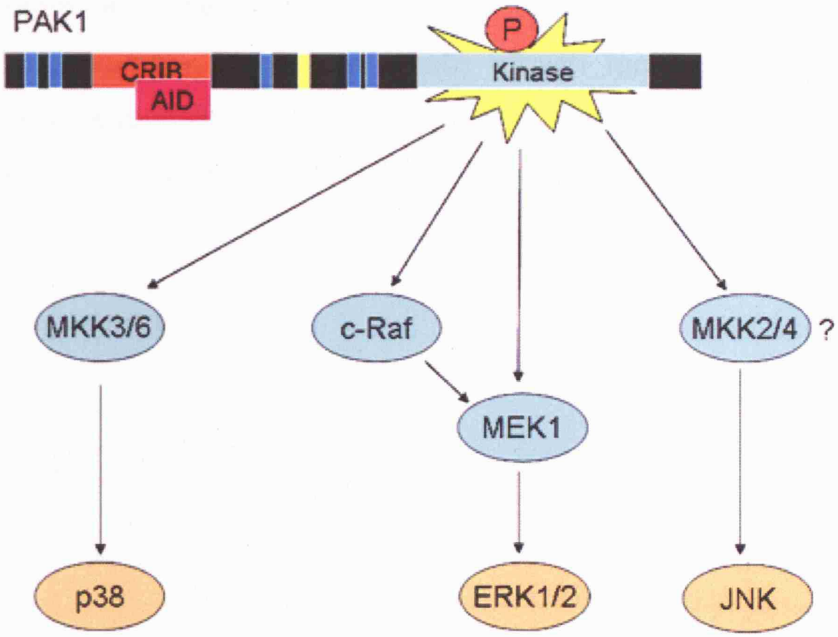



**Figure 1.14:** PAK1-mediated regulation of the MAPK pathways.
Diagram showing potential PAK1-mediated regulation of p38, ERK and JNK via the MAP kinase kinases and c-Raf.

MAPK activation is traditionally linked to transcriptional regulation, however, a number of other targets have been identified. Both p38 and ERK have been implicated in regulating cell motility downstream of PAK (Arai et al., 2005; Dechert et al., 2001; Lee et al., 2001; Rousseau et al., 2006) whilst JNK also regulates cell migration through phosphorylation of paxillin (Huang et al., 2004) although a direct link with PAK in this process has not been identified. Other roles for MAPK have also been identified without direct

evidence of activation by PAK. These include ERK-promoted sprouting during angiogenesis (Mavria et al., 2006), a process inhibited by a DN PAK1 peptide (Kiosses et al., 2002) and ERK mediated regulation of cell spreading (Ishibe et al., 2004), a process PAK1 regulates (Ten Klooster et al., 2006).

### 1.4: Haematopoiesis and macrophages

Haematopoiesis is the process of erythrocyte and leukocyte formation in the adult bone marrow. All mature cells within the blood derive from a single type of pluripotent stem cell which can differentiate down two possible pathways producing either lymphoid or myeloid stem cells. Determination of what pathway a stem cell follows and what mature differentiated cell it produces is mediated by a number of haematopoietic growth factors. These various cytokines are responsible for the regulation of survival, proliferation, differentiation and maturation of haematopoietic cells (Figure 1.15).

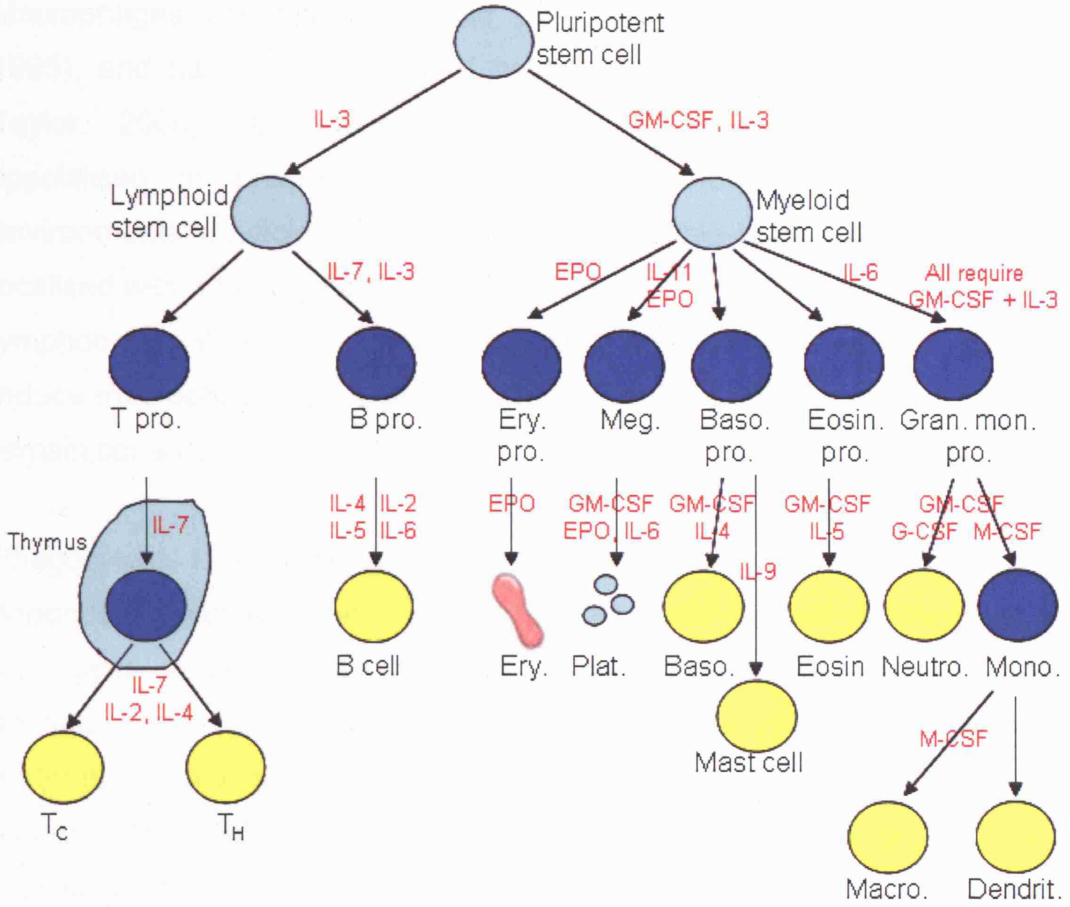



**Figure 1.15:** Cytokine regulation of haematopoiesis.
Stimulation of pluripotent stem cells by either activated macrophages, $T_H$ cells or by bone-marrow stromal cells induces haematopoietic activity and an increase in leukocyte population. Abbreviations: Baso. = basophil; Dendrit. = dendritic cell; Eosin. = eosinophil; Ery. = erythrocyte; EPO = erythropoietin; GM-CSF = granulocyte macrophage colony-stimulating factor; Gran. mon. = Granulocyte-monocyte; IL = interleukin; Macro. = macrophage; M-CSF = macrophage colony stimulating factor; Meg. = megakaryocyte; Mono = monocyte; Neutro = neutrophil; Plat. = platelets; Pro. = progenitor cell. Adapted from (Kuby, 1997).

### 1.4.1: The macrophage and its functions

It is upon monocyte extravasation from the blood vasculature into the tissues that circulating monocytes differentiate into mature cell types such as macrophages, dendritic cells and osteoclasts.

Macrophages become specialised depending on their location (Gordon, 1995), and have a high level of heterogeneity (see review (Gordon and Taylor, 2005)). Examples include alveolar macrophages which are specialised in phagocytic removal of micro-organisms, viruses and environmental particles in the lung whilst tingible-body macrophages are localised within the thymus and are responsible for phagocytosis of apoptotic lymphocytes. Whilst the surrounding microenvironment has the ability to induce macrophage heterogeneity, the underlying functions of macrophages remain conserved.

Phagocytosis is the process of actively taking up large particles in an actin-dependent manner. Macrophages phagocytose various micro-organism pathogens promoting their destruction within the lysosome. However, some bacteria, such as *M. tuberculosis*, can prevent complete phagosome maturation allowing their survival within a vacuole in the macrophage (Nguyen and Pieters, 2005). Macrophages phagocytose apoptotic cells in processes such as limb development and tissue remodelling (Hopkinson-Woolley et al., 1994). They are also responsible for phagocytosis of apoptotic fibroblasts (Moodley et al., 2003) and apoptotic neutrophils (Vivers et al., 2004) during wound healing (Peters et al., 2005).

Macrophages play a role in the regulation of inflammation during wound healing. Neutrophils, upon localising to a wound, typically induce an inflammatory response through cytokine release. Macrophage phagocytosis of apoptotic neutrophils reduced a number of inflammatory cytokine levels in the wound. Transforming growth factor-$\beta$ (TGF- $\beta$) levels, however, were increased suggesting macrophages inhibited the inflammatory response and induced cell growth (Fadok et al., 1998). Macrophages are capable of producing a number of cytokines which have exhibited differing functions in studies (Table 1.2). These include the pro-inflammatory cytokines IL-1, IL-6 and TNF$\alpha$ indicating that macrophages can both increase and decrease inflammation.

| | Endothelial Cell Proliferation | Angiogenesis | Fibroblast Proliferation | Collagen Synthesis |
|---|---|---|---|---|
| TNFα | ↑↓ | ↑ | ↑↓ | ↑ |
| IL-1 | ↓ | ↑↓ | ↑↓ | ↑ |
| IL-6 | ↓ | ↑ | ↓ | ↑ |
| TGF-β | ↑↓ | ↑ | ↑↓ | ↑ |
| TGFα | ↑↓ | ↑ | ↑↓ | ↑ |
| PDGF | ↑ | ↑ | ↑ | ↑ |
| IGF-1 | ↑ | ↑ | ↑ | ↑ |

**Table 1.2:** Some of the cytokines secreted by macrophages and their functions. Adapted from (Park and Barbul, 2004).

However, a number of disease states have deregulated macrophages implicated in their progression. Macrophages induce inflammation through cytokine production in response to cigarette smoke (Karimi et al., 2006) and an enhanced level of CSF-1 expression by the surrounding tissue, in conjunction with RANKL signalling, promotes macrophage differentiation into osteoclasts increasing bone destruction and the onset of rheumatoid arthritis (Danks et al., 2002). CSF-1-induction of macrophages has also been implicated in the auto-immune disease Lupus (see review (Chitu and Stanley, 2006)).

Interestingly, a role for macrophages in cancer has been identified (see review (Condeelis and Pollard, 2006)). A synergistic paracrine loop between mammary cancer cells and macrophages was established where CSF-1 secretion by the cancer cells and EGF secretion by the macrophages cooperatively enhanced their cell migration. It was suggested that macrophages are required for mammary tumour cell migration implicating macrophages in metastasis (Wyckoff et al., 2004). This theory is supported by the fact that macrophages can constitute a large part of a tumour's mass and this is associated with poor prognosis (see review (Bingle et al., 2002)). However, recent data also implicates macrophage association with tumours in the process of intravasation (Yamaguchi et al., 2006) and for the first time, angiogenesis *in vivo* (Bingle et al., 2006). It is apparent, therefore, that macrophages as well as being essential for the immune system, are capable

of inducing a number of disease states when the regulatory mechanisms are disturbed.

### 1.4.2: Colony stimulating factor-1

CSF-1 (M-CSF) is a pleiotropic cytokine that is required for macrophage survival, proliferation and differentiation. CSF-1-null mice show a decrease in tissue macrophages and osteoclasts which leads to osteopetrosis, skeletal abnormalities and tooth eruption failure (Cecchini et al., 1994; Wiktor-Jedrzejczak et al., 1990). However, CSF-1-independent macrophages have also been identified in certain tissues in CSF-1- and CSF-1R-null mice (Dai et al., 2002).

Three functionally separable types of CSF-1 have been identified, two secreted; a glycoprotein and proteoglycan, both of which circulate in the body, and one which is a cell-surface glycoprotein. The CSF-1 isoforms appear to have different functions. Expression of cell-surface CSF-1 (csCSF-1) in CSF-$1^{-/-}$ mice abated the growth retardation, tooth eruption and reproductive defects seen in null mice and normal macrophage numbers were observed in some tissues. However, osteopetrosis defects and hematologic defects in the peripheral blood, bone marrow and spleen were not recovered with the expression of csCSF-1 suggesting different CSF-1 isoforms perform different functions *in vivo* (Dai et al., 2004). The three alternatively spliced mRNAs that encode the CSF-1 isoforms have been identified in all the cell types studied and little has been determined about their differential regulation.

### 1.4.3: The CSF-1 receptor

The colony-stimulating factor-1 receptor (CSF-1R) is encoded by the proto-oncogene *c-fms* and is essential for macrophage differentiation but is also expressed in osteoclasts, dendritic cells (MacDonald et al., 2005), prostate cells (Ide et al., 2002) and breast cancer cells (Kluger et al., 2004). The CSF-1R contains an extracellular ligand-binding domain, a transmembrane

domain and an intracellular tyrosine kinase domain which autophosphorylates upon CSF-1 stimulation and receptor dimerisation (see review (Pixley and Stanley, 2004)). Upon dimerisation and autophosphorylation, an extracellular disulphide bond is formed to covalently link the receptors allowing further phosphorylation of the receptor. Dimerisation promotes activation of a number of signalling pathways before the receptor is downregulated through internalisation and degradation (see review (Pixley and Stanley, 2004)).

### 1.4.4: CSF-1 signalling and macrophage proliferation

Studies have shown that CSF-1 is required for macrophage survival and proliferation (Cecchini et al., 1994; Dai et al., 2002) although lower concentrations are required to promote survival than for proliferation (Tushinski et al., 1982). The signalling pathways involved in this process are not entirely clear but it has been established that PI3-K is required for macrophage survival but not proliferation (Murray et al., 2000) and that activation of ERK (Bhatt et al., 2002) and NF-$\kappa$B (Zhang et al., 2003) is downstream of PI3-K and help regulate the survival response. The pathways responsible for macrophage proliferation remain unclear but are potentially through JNK activation (Himes et al., 2006) (Figure 1.16).

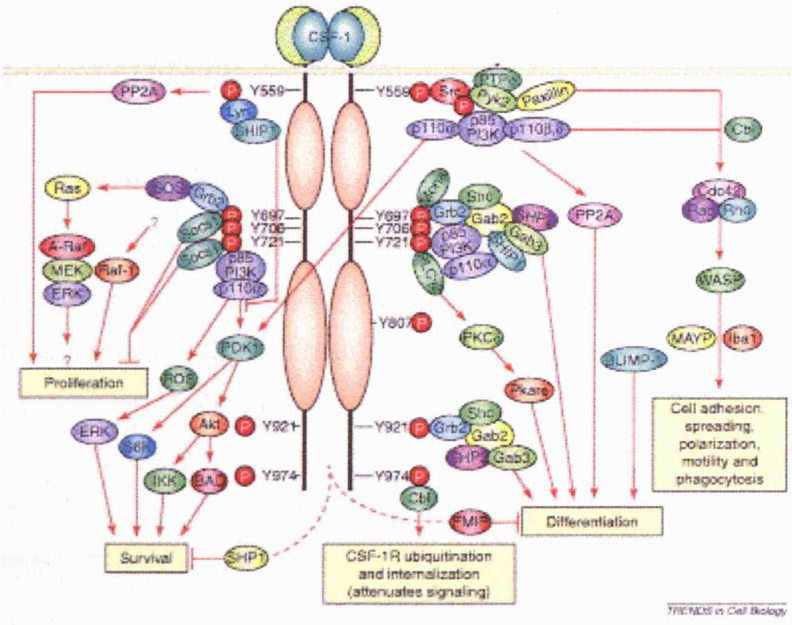


**Figure 1.16:** The signalling pathways of CSF-1R after stimulation in myeloid cells. Taken from (Pixley and Stanley, 2004).

### 1.4.5: CSF-1 and macrophage migration

Migration is essential for macrophage function and is required for the extra-vasation of the macrophage precursor monocytes. Stimulation of quiescent macrophages with CSF-1 induces migration through promotion of lamellipodia formation, membrane ruffling and polarisation, processes which all require cytoskeletal reorganisation. Ultimately chemotaxis towards the source of CSF-1 is induced (Allen et al., 1998; Boocock et al., 1989; Webb et al., 1996). CSF-1R regulation of PI3-K increases the $PIP_3$ levels at the membrane upon stimulation, inducing the binding of a number of pleckstrin homology (PH) domain containing proteins. Potential proteins recruited to these sites include the GEFs which activate the Rho GTPases. As has already been stated, activation of the Rho GTPases can induce cytoskeletal reorganisation and migration (Chapter 1.2.2.4). Rac activation and induction of spreading and ruffling has been observed upon CSF-1 stimulation of macrophages (Allen et al., 1997; Wells et al., 2004) whilst Cdc42 induction of filopodia formation was also shown to be required for macrophage chemotaxis (Allen et al., 1998). A downstream effector of Cdc42 during chemotaxis appears to be WASp because CSF-1-induced chemotaxis was abolished in Wiscott-Aldrich syndrome (WAS) macrophages but was unaffected in neutrophils (Zicha et al., 1998).

Macrophage migration also involves the regulation of adhesions. CSF-1 stimulation of CSF-1R has been directly implicated in adhesion formation and turnover although macrophages only produce smaller focal contacts and not the larger focal adhesions observed in fibroblasts. However, a number of the same proteins are localised in the focal contacts. Pyk2 is a non-receptor tyrosine kinase closely related to FAK which has been shown to be phosphorylated by Src upon CSF-1 stimulation (see review (Pixley and Stanley, 2004)). Phosphorylated Pyk2 is capable of binding paxillin and has been shown to be localised within adhesions with integrins and CSF-1R

(Elsegood et al., 2006) suggesting Pyk2 signalling regulates these structures and cell adhesion. Further evidence for Pyk2 regulation of adhesion is shown from reports indicating that lipopolysaccharide (LPS) induces Pyk2 and paxillin phosphorylation (Williams and Ridley, 2000) whilst DN Pyk2 inhibited paxillin phosphorylation (Hiregowdara et al., 1997). The importance of this is indicated by $Pyk2^{-/-}$ macrophages which had a reduced ability to polarise and retract its migratory tail (Okigaki et al., 2003).

Macrophages and osteoclasts have podosomes, a different type of adhesion which is made of an F-actin core surrounded by a ring of integrins and associated proteins such as paxillin (see review (Linder and Kopp, 2005)). CSF-1 stimulation of macrophages increases podosome formation in a PI3-K dependent manner (Wheeler et al., 2006a) and Pyk2 has been shown to localise to podosomes (Duong and Rodan, 2000). Roles for PAK1 and cortactin have also been identified for podosome formation in smooth muscle cells (Webb et al., 2005; Webb et al., 2006; Zhou et al., 2006) whilst WAS macrophages have no podosomes identifying a role for WASp in podosome formation (Jones et al., 2002).

## 1.5: Aims

PAKs have been implicated in a number of signalling pathways and cellular processes. However, many of the reports in the literature have used over-expression studies of CA and DN PAK mutants to determine roles for the PAK proteins. Over-expression studies, although useful, may swamp the system and produce artificial results through effector sequestering and interference with protein localisation and compartmentalisation. The aim of this project is therefore, to investigate the specific role of PAK1 in macrophage signalling, spreading and migration.

Study of PAK1 function was enabled through use of a PAK1-null mouse generated by the Chernoff laboratory (Ten Klooster et al., 2006). Unfortunately, it was not possible to compare data gained from the PAK1-null mouse with a PAK2-null mouse as deletion of PAK2 was shown to be embryonically lethal (Dr. J. Chernoff, personal communication). However, comparison of PAK1-null cells with WT cells should allow the determination of PAK1 specific roles.

To study PAK1 function, bone marrow-derived macrophages (BMMs) were used as the model system. BMMs were chosen because they migrate as single cells and this is regulated by Rho GTPases. They can also be derived from mice, purified and stored allowing experimental repetition with cells from the same source.

# Chapter 2: Methods.

## 2.1: General reagents

| Reagent | Source | Address |
|---|---|---|
| Ammonium persulphate | Sigma-Aldrich | www.sigmaaldrich.com |
| β-mercaptoethanol | Sigma-Aldrich | www.sigmaaldrich.com |
| BioRad protein assay reagent | BioRad laboratories | www.bio-rad.com |
| Bovine serum albumin | Sigma-Aldrich | www.sigmaaldrich.com |
| Coomassie brilliant blue (R250) | BioRad laboratories | www.bio-rad.com |
| Recombinant murine CSF-1 | R&D systems | www.rndsystems.com |
| DAKO mounting medium | DAKO-cytomation Inc. | www.dakocytomation.us |
| Dimethylsulphoxide (DMSO) cat. No. D2650 | Sigma-Aldrich | www.sigmaaldrich.com |
| 50 Bp DNA ladder | Invitrogen | www.invitrogen.com |
| Dried skimmed milk (Marvel) | Tesco | www.tesco.com |
| ECL (enhanced chemi-luminescence kit) | Amersham Biosciences | www.amershambiosciences.com |
| Endo-free maxi prep kit | Qiagen | www.qiagen.com |
| Multimark protein size markers | Invitrogen | www.invitrogen.com |
| NuPage protein electrophoresis system | Invitrogen | www.invitrogen.com |
| Oligonucleotides | Sigma-Genosys | www.sigma-genosys.com |
| PageRuler pre-stained protein ladder | Fermentas Life Sciences | www.fermentas.com |
| Pre-developed TaqMan assay reagents | Applied Biosystems | www.appliedbiosystems.com |
| Rainbow coloured protein markers (RPN 756) | Amersham Biosciences | www.amershambiosciences.com |
| Reverse transcription kit | Applied Biosystems | www.appliedbiosystems.com |
| RNeasy RNA extraction kit | Qiagen | www.qiagen.com |
| Super RX medical X-ray film | Fuji film | www.fujifilm.com |
| Superscript RT-PCR kit | Invitrogen | www.invitrogen.com |
| Sure2 super-competent bacteria | Stratagene | www.stratagene.com |

| | | |
|---|---|---|
| Taq DNA polymerase kit | Invitrogen | www.invitrogen.com |
| Taqman Universal PCR mix | Applied Biosystems | www.appliedbiosystems.com |
| Taqman primers and probes | Applied Biosystems | www.appliedbiosystems.com |
| Recombinant TNF*α* | Serotec | www.serotec.com |
| Transwell filters | Corning | www.corning.com |

## 2.2: Cell Culture

### 2.2.1: Materials

| | | |
|---|---|---|
| Dulbecco's modified eagle medium | Invitrogen | www.invitrogen.com |
| Foetal calf serum (FCS) | Helena-Bioscience | www.helena-biosciences.com |
| Goat serum | Invitrogen | www.invitrogen.com |
| Penicillin/streptomycin | Invitrogen | www.invitrogen.com |
| Phosphate buffered saline (PBS) | Invitrogen | www.invitrogen.com |
| 100x Non-essential amino acids | Invitrogen | www.invitrogen.com |
| RPMI 1640 + 1% glutamine | Invitrogen | www.invitrogen.com |
| 100 mM Sodium pyruvate | Invitrogen | www.invitrogen.com |
| Trypsin/EDTA | Invitrogen | www.invitrogen.com |
| Versene (PBS + 0.48 mM EDTA) | Invitrogen | www.invitrogen.com |

### 2.2.2: Cell culture medium

i) Macrophage growth medium

RPMI 1640 medium containing L-glutamine, supplemented with 10% (v/v) FCS, heat-inactivated by incubation for 30 minutes at 65°C, 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids, 10 μM β-mercaptoethanol, 100 IU/ml Penicillin and 100 μg/ml Streptomycin. 10% L-cell conditioned medium was added as a source of CSF-1.

ii) Macrophage starve medium

Macrophage growth medium without L-cell conditioned medium.

iii) Macrophage freezing medium

Macrophage starve medium supplemented with 10% DMSO.

iv) L-cell conditioned medium

L-cells were grown to confluency in DMEM containing 10% heat-inactivated FCS, 100 IU Penicillin and 100 μg/ml Streptomycin. The lids of the flasks containing the cells were sealed and the cells were incubated for 3 weeks at 37°C, 10% $CO_2$. The L-cell medium was sterilized by filtration through a 0.25 μm filter and stored at 4°C for up to 6 months. L-cell conditioned medium was kindly prepared by R. Garg and P. Bhavsar.

v) Cos7 medium

DMEM supplemented with 10% FCS (v/v) and 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.2.3: Extraction of bone marrow cells and macrophage differentiation

Femoral bone marrow cells were isolated from 6-8 week old C57B6 (black 6) mice (WT). Briefly, the femur was dissected from the hind leg and scraped clean. Holes were pierced into either end of the bone and the bone marrow was washed out with macrophage starve medium. Bone marrow cells from $PAK1^{-/-}$ and WT mice with a C57B6/Sv129 or a C57B6 genetic background generated in Jonathan Chernoff's laboratory (Fox Chase Cancer Centre, Philadelphia, USA) were also used. The bone marrow cells were seeded at $1x10^6$ cells/ml into 6-cm bacteriological plates (Falcon) in macrophage growth medium. The cells were incubated in a humidified incubator at 37°C with 10% $CO_2$ for 3 days. The non-adherent population was then collected and cryogenically frozen in macrophage freezing medium at $1x10^6$ cells/ml in cryotubes. Upon thawing, cells were incubated for a further 4 days in macrophage growth medium on bacterial plates to produce a relatively homogenous population of adherent macrophages.

2.2.4: Culture of bone marrow macrophages (BMMs)

BMMs were cultured in macrophage growth medium and the medium was changed every 3 days. To passage BMMs, the cells were washed once with

PBS and incubated in versene for 5 minutes at 37°C and 10% $CO_2$. To aid detachment of cells, versene was pipetted over the cells. Upon detachment of all BMMs from the bacteriological plastic, macrophage medium was added to the versene in a 1:1 dilution. Cells were collected by centrifugation at 1,100 rpm (ALC PK130R) for 5 minutes and replated in macrophage growth medium. BMMs were not used for more than 14 days after thawing.

### 2.2.5: Starvation and stimulation of BMMs

Growth medium was removed and replaced with macrophage starve medium and cells were incubated for 16 hours. BMMs were stimulated using either 33 ng/ml CSF-1 or 10 ng/ml TNFα in macrophage starve medium for the time points indicated on the figures.

### 2.2.6: Treatment of BMMs with inhibitors

Before treatment with chemical inhibitors, BMMs were washed with PBS. Macrophage growth medium containing the inhibitor was added to the BMMs and incubated for the time indicated before being lysed or plated for filming.

| Inhibitor | Target | Final Concentration | Incubation Time |
|---|---|---|---|
| ALLnL | Proteasome | 10 μg/ml | 24 hr |
| Cycloheximide | Protein Translation | 10 μg/ml | 0-4 hr |
| Lactacystin | Proteasome | 10 μg/ml | 24 hr |
| U0126 | MEK | 1 μg/ml | 1 hr |

**Table 2.1:** Inhibitors used on BMMs.

2.2.7: Stimulation of translation-inhibited BMMs

To evaluate PAK1 responses upon CSF-1 stimulation in protein translation-inhibited cells, 10 μg/ml cycloheximide was added to macrophage starve medium containing 33 ng/ml CSF-1.

2.2.8: Culture of Cos7 cells

Cos7 cells were grown in Cos7 medium in 10-cm tissue culture dishes in a humidified atmosphere incubator at 37°C with 10% $CO_2$. To passage, the cells were washed in PBS and then incubated with 3 ml trypsin/EDTA at 37°C and 10% $CO_2$ until cells had detached from the dish. Cos7 medium was added to inactivate the trypsin and the cells were collected by centrifugation at 1000 rpm (ALC PK130R) for 5 minutes. Cells were seeded onto fresh dishes at dilutions according to their growth rate, typically between 1:5 and 1:20 in Cos7 medium.

## 2.3: Protein biochemistry

2.3.1: Buffers and solutions

| | |
|---|---|
| Coomassie Blue destain | 15% Methanol (v/v)<br>5% Acetic acid (v/v) |
| Coomassie blue stain | 15% Methanol (v/v)<br>5% Acetic acid (v/v)<br>0.025% Coomassie blue (w/v) |
| MOPS running buffer | 50 mM morpholinepropanesulfonic acid<br>50 mM Tris-Cl pH 7.6<br>0.1% SDS (w/v)<br>1 mM EDTA pH 8.0 |
| 1% NP-40 lysis buffer | 50 mM Tris-Cl pH 7.6<br>2 mM EDTA pH 8.0<br>150 mM NaCl<br>10 mM $MgCl_2$<br>1% NP-40<br>10% glycerol (v/v)<br>10 μg/ml DTT |

| | |
|---|---|
| | Protease inhibitor cocktail |
| Protease inhibitor cocktail | 1 μg/ml Leupeptin<br>1 mM PMSF<br>1 mM Sodium Vanadate<br>0.72 IU Aprotinin<br>1 mM Sodium fluoride |
| 10x SDS-PAGE running buffer | 250 mM Tris-Cl<br>4% SDS (w/v)<br>1.92 M Glycine |
| 2x SDS-PAGE sample buffer | 0.125 M Tris-Cl pH 6.8<br>4% SDS (w/v)<br>20% Glycerol (v/v)<br>1.41 M β-mercaptoethanol |
| SDS-PAGE separation gel | 3.75 mM Tris-Cl pH 8.8<br>7.5, 10 or 12% Acrylamide (w/v)<br>1% SDS (w/v)<br>0.05% APS (w/v)<br>0.005% Temed (v/v) |
| SDS-PAGE stack gel | 630 μM Tris-Cl pH 6.8<br>10% Acrylamide (w/v)<br>0.5% APS (w/v)<br>0.1% Temed (v/v) |
| 10x SDS- PAGE transfer buffer | 250 mM Tris-Cl<br>1.92 M Glycine<br>(20% methanol added during dilution) |
| Strip buffer | 62.5 mM Tris-Cl pH 6.8<br>2% SDS (w/v)<br>0.7% β-mercaptoethanol (v/v) |
| TBS 0.5% Tween | 25 mM Tris-Cl pH 7.6<br>50 mM NaCl<br>0.5% Tween 20 (v/v) |

2.3.2: Antibodies used in western blotting

| Primary Antibodies | | | | |
|---|---|---|---|---|
| Antibody | Reactivity | Dilution | Source | Address |
| β-actin | Mouse | 1/5000 | Sigma-Aldrich | www.sigmaaldrich.com |
| Cyclin D1 | Mouse | 1/1000 | Santa-Cruz Biotechnology | www.scbt.com |
| ERK 1/2 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| GEF-H1 | Rabbit | 1/500 | Gift-Maria Balda | - |

| | | | | |
|---|---|---|---|---|
| NET1 | Goat | 1/500 | Santa-Cruz Biotechnology | www.scbt.com |
| PAK1 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| PAK 1/2/3 | Rabbit | 1/1000 | Santa-Cruz Biotechnology | www.scbt.com |
| PAK3 | Rabbit | 1/1000 | Chemicon | www.chemicon.com |
| p-Akt Ser473 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-c-Raf Ser338 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-ERK 1/2 Thr202/Tyr204 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-JNK Thr183/Tyr185 | Rabbit | 1/1000 | New England Biolabs | www.neb.com |
| p-LIMK 1/2 Thr505/Thr508 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-MEK1 Ser298 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-MLC2 Ser19 | Mouse | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| p-Op18 Ser16 | Rabbit | 1/1000 | Santa-Cruz Biotechnology | www.scbt.com |
| p-p38 Thr180/Tyr182 | Rabbit | 1/1000 | New England Biolabs | www.neb.com |
| p-Paxillin Tyr118 | Rabbit | 1/1000 | Biosource | www.biosource.com |
| p-PAK 1/2 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| $\beta$-PIX | Rabbit | 1/1000 | Chemicon | www.chemicon.com |
| p-Pyk2 Thr402 | Rabbit | 1/1000 | Cell Signalling Technology | www.cellsignal.com |
| $\beta$-tubulin | Mouse | 1/2000 | Sigma-Aldrich | www.sigmaaldrich.com |
| **Secondary Antibodies** | | | | |
| **Antibody** | | **Dilution** | **Source** | **Address** |
| Horse radish peroxidase (HRP)-conjugated anti-Mouse | | 1/4000 | Amersham Biosciences | www.amersham.com |
| Horse radish peroxidase (HRP)-conjugated anti-Rabbit | | 1/2000 | Amersham Biosciences | www.amersham.com |
| Horse radish peroxidase (HRP)-conjugated anti-Goat | | 1/2000 | Santa-Cruz Biotechnology | www.scbt.com |

**Table 2.2:** Primary and secondary antibodies used in western blotting.

2.3.3: Cell lysis

BMMs were grown to approximately 80% confluence on tissue culture plastic dishes and stimulated as required experimentally. Cells were placed on ice, washed twice with PBS and lysed in an appropriate volume of NP-40 lysis buffer (cells grown on 2-cm dishes were lysed in 200 µl of lysis buffer). Lysates were incubated in eppendorf tubes on ice for 15-30 minutes before

centrifugation at 13,000 rpm (Eppendorf 5417R) for 10 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube and the insoluble pellet discarded. The protein concentration was determined using the BioRad protein assay, then 2x sample buffer was added and the samples boiled at 100°C for 3 minutes. The samples were stored at -20°C.

2.3.4: BioRad protein assay

A 5 µl sample of cell lysate was added to 795 µl of $ddH_2O$ and 200 µl of BioRad protein assay dye. A blank was made by adding 5 µl of lysis buffer rather than protein sample and the solutions were mixed by inversion. The absorbance of each sample was measured at 595 nm in a plastic cuvette using a BioRad spectrophotometer.

2.3.5: SDS-PAGE

Proteins were separated using an SDS-PAGE mini-gel system (Mini-Protean Cell, BioRad). A gel of 1.5-mm thickness was cast, which contained 7.5, 10 or 12% acrylamide and overlaid with 20% ethanol. The gel was allowed to set at room temperature. Once the separation gel was set, a 10% acrylamide stacking gel was layered on top and a gel comb inserted to create wells for protein sample loading. The set gel was placed into a gel tank containing 1x SDS-PAGE running buffer and electrophoresed at 120 volts until the Coomassie blue dye in the sample buffer ran from the bottom of the gel.

2.3.6: NuPAGE electrophoresis

For separation of proteins similar in molecular weight, NuPAGE electrophoresis was used. This system uses pre-cast Bis-Tris SDS-PAGE gels which have a 4-12% acrylamide gradient. Samples were added to the gel and electrophoresed in an X-Cell II gel tank containing fresh 1x MOPS running buffer at 150 volts for 1 hour. The gels were then western blotted as for a normal SDS-polyacrylamide gel.

2.3.7: Western blotting

Upon completion of protein separation by gel electrophoresis, proteins were transferred onto either polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Schleicher & Schuell) or nitrocellulose membrane (Protran, Millipore) by wet transfer. Polyacrylamide gels were overlaid with PVDF (pre-soaked for 1 minute in methanol) or nitrocellulose (briefly pre-soaked in 1x transfer buffer) membrane. The gel and transfer membrane were sandwiched between two pieces of Whatmann 3-mm paper which had been soaked in 1x transfer buffer. Air bubbles were removed from between the gel and membrane and placed between two sponges that had been pre-soaked in 1x transfer buffer. This was then placed in a Mini Protean II transfer case and inserted into a gel tank containing 1x transfer buffer pre-cooled to 4°C. Proteins were transferred at 4°C and 100 volts for 1 hour. The transfer membrane was blocked in 5% non-fat dried milk or 5% BSA (depending upon antibody to be used) for 1 hour at RT with agitation. The membrane was washed 4 times in TBS 0.5% Tween and incubated with 0.1-0.5 μg/ml primary antibody in the appropriate block for either 1 hour at RT or overnight at 4°C with agitation. The membrane was washed 4 times in TBS 0.5% Tween and incubated with the appropriate HRP-conjugated secondary antibody in 1% non-fat dried milk or BSA for 1 hour at RT with agitation. The membrane was again washed 4 times in TBS 0.5% Tween and developed in ECL for 1 minute at RT. The membrane was covered in Saran wrap and exposed to X-ray film in the dark.

2.3.8: Stripping western blots

Transfer membranes were placed in 10 ml of strip buffer (β-mercaptoethanol added just before use) and incubated at 65°C for 20 minutes with agitation. Membranes were washed 5 times with TBS 0.5% Tween and re-blocked with 5% non-fat dried milk or 5% BSA for 1 hour at RT. The membrane was then probed according to the standard western blotting procedure.

2.3.9: Quantification of western blots

Western blot autoradiographs were scanned using a BioRad GS-800 Densitometer and the BioRad software Quantity One. Bands of interest were analysed by densitometry to give a numerical output. Bands were normalised to loading controls and data were analysed using Microsoft Excel.

## 2.4: Molecular biology

2.4.1: Materials

| | |
|---|---|
| Ampicillin | 50 mg/ml stock in $ddH_2O$ |
| DEPC-treated water | 1% DEPC (v/v), incubated overnight then autoclaved |
| 6x DNA loading buffer | 30% Glycerol<br>0.25% Bromophenol blue (w/v)<br>0.25% Xylene cyanol (w/v) |
| IPTG | 0.5 M stock in $ddH_2O$ |
| LB | 25g per litre of Premix |
| LB Agar | 15g per litre Bacto agar added to LB |
| LB/Amp | LB supplemented with 50 μg/ml ampicillin |
| 10x TBE buffer | 960 mM Tris<br>890 mM Boric acid<br>20 mM EDTA |
| T.E. pH 8 | 100 mM Tris Cl<br>10 mM EDTA (pH 8.0) pH adjusted if needed using 1 M NaOH or 4 M HCl |

2.4.2: Plasmids

| | |
|---|---|
| pcDNA-PAK1-myc | Gift from Dr P. Burbelo |
| pPAK2-myc | Ridley laboratory |
| pJ3 PAK3-HA | " " |
| pSR*α*-PAK4-HA | " " |
| pCMV6M-PAK5-Myc | Gift from Dr J. Chernoff |
| pCNA3-Flag-PAK6 | Gift from Dr G. Bokoch |

2.4.3: Primers

| *Primer Name* | *Sequence* |
|---|---|
| PAK1 forward 254 | CTATGATTGGAGCCGGCAGC |
| PAK1 reverse 1183 | CCAGGTAGTTGACAATATTTGG |
| PAK2 forward 159 | CATCTCCATATTCTCTGGCA |
| PAK2 reverse 1207 | TAGTACTGCGTTTGCTCTGT |
| PAK3 forward 1008 | TCCTTTGGCTCCTCCTGTATC |
| PAK3 reverse 1646 | TCACTTGGTTTGAGTGCAAG |
| PAK4 forward 300 | GAGCAAGGAGGTGCCTCGGA |
| PAK4 reverse 514 | TCTTCAGTCGGGGCGGGAGG |
| PAK5 forward 257 | TAGAGGATTTTGACAACATCTCC |
| PAK5 reverse 867 | TTGAGGTAGTTTGGTGGGGC |
| PAK6 forward 73 | CACACCTCCTTTGACCCCAAG |
| PAK6 reverse 539 | ACGTAGCTGTCCAGCAGCAG |
| $\beta$-actin forward | CCAACCGTGAAAAGATGACC |
| $\beta$-actin reverse | AATTGAATGTAGTTTCATGGATG |

2.4.4: Determination of RNA/DNA concentration

The concentration of RNA and DNA within a solution was determined by placing a sample in a quartz cuvette and measuring the optical density at 260 nm and 280 nm using a BioRad spectrophotometer. The concentration of DNA and RNA was calculated as follows:

$Conc_{DNA}$ mg/ml = OD 280 x 50

$Conc_{RNA}$ mg/ml = OD 260 x 40

2.4.5: Isolation of total cellular RNA and cDNA production

For identification of the BMMs PAK expression profile, the following RNA extraction protocol was used. A confluent 10-cm dish of BMMs was lysed with 2 ml of Trizol reagent (Invitrogen), scraped and 1 ml was placed into two 1.5 ml eppendorf tubes. This was incubated at RT for 5 minutes. 0.2 ml of

chloroform was added, mixed vigorously for 15 seconds and then incubated at RT for 3 minutes. The samples were centrifuged at 12,000 rpm (Eppendorf 5417R) for 15 minutes at 4°C. After centrifugation, the upper aqueous phase was transferred to a new eppendorf tube and 0.5 ml of -20°C isopropanol was added and mixed by inversion. This was incubated at RT for 10 minutes and then centrifuged at 12,000 rpm (Eppendorf 5417R) for 15 minutes at 4°C to pellet the RNA. The pellet was washed once with 1 ml of -20°C 75% ethanol and centrifuged at 7,500 rpm (Eppendorf 5417R) for 5 minutes at 4°C and allowed to air dry. The pellet was resuspended in diethylpyrocarbonate (DEPC) $H_2O$ (0.1% DEPC in $H_2O$, mixed overnight and autoclaved) and incubated at 55-60°C until completely dissolved.

For cDNA synthesis the Superscript RT-PCR kit was used. Briefly, total cellular RNA was DNase I-treated for 15 minutes at RT to remove any residual genomic DNA. DNase I was inactivated by addition of 25 mM EDTA and incubating at 65°C for 10 minutes. The RNA was primed with the addition of oligo-dT primers and 10 mM dNTP mix at 65°C for 5 minutes and then chilled on ice. 5x $1^{st}$ strand buffer, 0.1 M DTT and the RNase inhibitor, RNase out, were added to the sample at 42°C for 2 minutes. Reverse transcriptase was then added and incubated for 50 minutes at 42°C before inactivation through incubation at 70°C for 5 minutes and determination of the cDNA concentration.

### 2.4.6: Design of PCR primers

The *Mus musculus* genome was searched for the PAKs using the nucleotide programme which is part of the NIH Pubmed website (http://www.ncbi.nlm.nih.gov/entrez/). cDNAs for PAK1 and PAK3 were found whilst the genes for PAK2, 4, 5 and 6 were identified from the mouse genome (http://www.ensembl.org/Mus_musculus/). 20-22mers of PAK1-PAK6 were chosen for RT-PCR primers and were then searched in the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure that the sequences chosen were unique for each PAK.

2.4.7: Polymerase chain reaction

Amplification of cDNA by PCR was carried out using the Taq DNA polymerase kit. Briefly, 10 to 50 ng of template cDNA, 40 μM of forward and reverse primers, 200 μM of each dNTP, 2 μM $MgCl_2$ in a total of 20 μl of 1x PCR buffer was used. 0.4 μl of Taq polymerase was added prior to the start of the reaction. Initially, the DNA was denatured by heating each reaction to 95°C for 2 minutes before 30 cycles of the following programme were run:

Denaturation: 95°C – 30 seconds
Annealing: 57°C (PAK1), 59°C (PAK2-6) – 30 seconds
Elongation: 72°C – 1 minute

A final elongation of 72°C for 5 minutes and a cooling to 15°C followed the cycles. Each PCR reaction had a positive control using the plasmids encoding PAK1-6 listed in Chapter 2.4.2. The PCR products were analysed by agarose gel electrophoresis.

2.4.8: Agarose gel electrophoresis

PCR products were analysed by size separation on 1.5% TBE agarose gels. Agarose gels were prepared by addition of 1.5g of electrophoresis grade agarose resuspended in 100 ml of TBE which was dissolved by boiling in a microwave oven. After cooling, 0.5 μg/ml ethidium bromide was added to allow visualisation of PCR products under UV illumination (254 nm). A gel cassette was sealed with masking tape, a well comb inserted and then the gel poured and allowed to set. The set gel was inserted into a gel tank containing 1x TBE running buffer, the well comb was removed and a DNA sample mixed with 6x DNA loading buffer was added to each well. 5 μl of 50 b.p DNA ladder was added to the first well to determine the size of the DNA products on the gel. The DNA was electrophoresed across the gel at 100 volts until the xylene cyanol dye in the loading buffer reached the edge of the

gel. The gel was photographed using a dual intensity UV transillumination box (GRI) and a Syngene camera (Sony).

2.4.9: Taqman quantitative RT-PCR

BMMs were seeded onto 6-well tissue culture dishes at $2x10^5$ cells/well and grown for 24 hours. BMMs were starved of CSF-1 for 16 hours and then stimulated with either 33 ng/ml CSF-1 or 10 ng/ml TNFα for the times stated in the figures. After stimulation, the cells were lysed and the RNA extracted using the RNeasy RNA extraction kit as in the manufacturer's instructions. The RNA was DNase treated during the purification to digest any genomic DNA present.

Using the Applied Biosciences reverse transcription kit, cDNA was made from the BMMs RNA for each stimulation time point and a series dilution between undiluted and 1/10,000 dilution was performed. For Taqman quantitative PCR, 10 μl of mastermix (5 μl mastermix buffer, 2.5 μl DEPC water (Ambion), 2 μl cDNA and 0.5 μl probe) was added to the wells of a 384 well plate. For each experiment, an 18s rRNA probe was used as a control for cDNA loading and either siglec-1 or TNFα probe was used as a positive control of stimulation. PAK1 and PAK3 probes were used to determine any changes in their transcript levels. Each cDNA sample was run over the dilution series and each dilution in duplicate. The appropriate cDNA was added to each well and it was sealed and centrifuged at 350 rpm (ALC PK130R) for 1 minute at RT. The plate was then loaded into the ABI prism 7900 HT sequence detection system and the following programme was run:

50°C – 2 min
95°C – 10 min
95°C – 15 sec
60°C – 1 min } x 40 cycles

Upon completion of the run, a graphical and numerical output was produced which was analysed in Microsoft Excel.

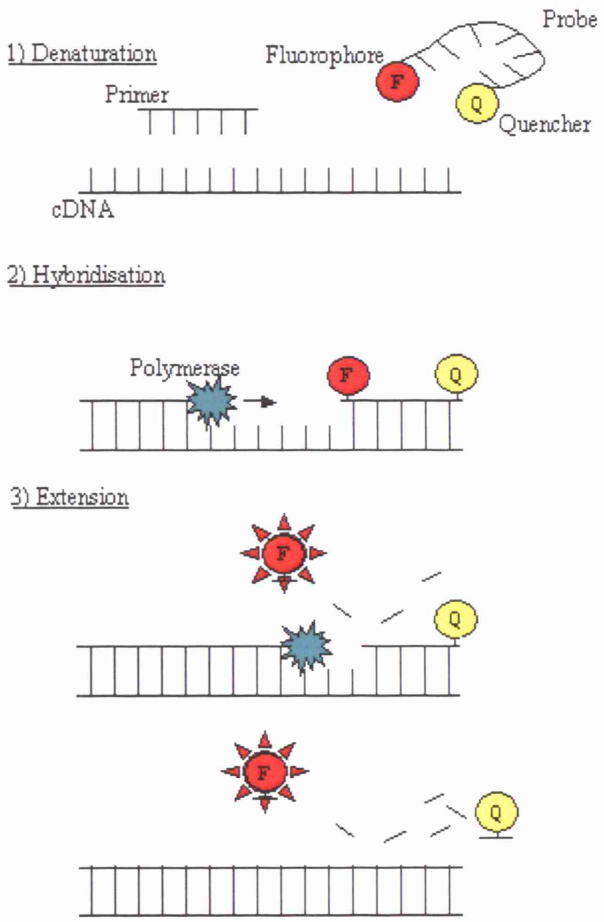



**Figure 2.1:** Diagram showing Taqman quantitative RT-PCR.

## 2.5: Cell Biology

### 2.5.1: Materials

| Primary Antibodies for Immunofluorescence | | | | |
|---|---|---|---|---|
| Antibody | Reactivity | Dilution | Source | Address |
| $\beta$-PIX | Rabbit | 1/500 | Chemicon | www.chemicon.com |
| $\beta$-tubulin | Mouse | 1/250 | Sigma-Aldrich | www.sigmaaldrich.com |
| ERK 1/2 | Rabbit | 1/100 | Cell Signalling Technology | www.cellsignal.com |
| FITC conjugated $\alpha$-tubulin | Mouse | 1/200 | Sigma-Aldrich | www.sigmaaldrich.com |
| PAK1 | Rabbit | 1/50 | Cell Signalling Technology | www.cellsignal.com |
| PAK1 | Rabbit | 1/100 | Sigma-Aldrich | www.sigmaaldrich.com |

| | | | | |
|---|---|---|---|---|
| PAK2 | Rabbit | 1/200 | Cell Signalling Technology | www.cellsignal.com |
| P-ERK 1/2 Thr202/Tyr204 | Rabbit | 1/200 | Cell Signalling Technology | www.cellsignal.com |
| Rac | Rabbit | 1/200 | Upstate | www.upstate.com |
| Secondary Antibodies | | | | |
| Antibody | | Dilution | Source | Address |
| Cy5 conjugated anti-rabbit | | 1/200 | Jackson Immuno-research | www.jacksonimmuno.com |
| FITC conjugated anti-mouse | | 1/200 | Southern Biotechnology Associates | www.southernbiotech.com |
| Mouse anti-GST | | 1/200 | Abcam | www.sigmaaldrich.com |
| Rabbit anti-GST | | 1/500 | Sigma-Aldrich | www.abcam.com |
| TRITC conjugated anti-mouse | | 1/200 | Southern Biotechnology Associates | www.southernbiotech.com |
| Non-antibody Reagents | | | | |
| Domain | Species | Dilution | Source | Address |
| GST-N-WASp CRIB | Human | 1/40 | Ridley laboratory | - |
| 0.05 mg/ml Rhodamine-conjugated phalloidin | Sigma-Aldrich | 1/500 | Sigma-aldrich | Sigma-Aldrich |

**Table 2.3:** Antibodies and peptides used in immunofluorescence.

2.5.2: Microscopes

| *Equipment* | *Manufacturer* | *Address* |
|---|---|---|
| Axiophot Microscope | Carl Zeiss | www.zeiss.com |
| Axiovert 135 Microscope | Carl Zeiss | www.zeiss.com |
| Eclipse TE 2000-E Microscope | Nikon | www.nikon-instruments.com |
| LSM510 Confocal microscope | Carl Zeiss | www.zeiss.com |
| KPM1E/K-S10 CCD camera | Hitachi Denshi | www.hdal.com |
| ORCA-ER CCD camera | Hamamatsu Photonics | www.hamamatsu.com |

2.5.3: Software

| *Equipment* | *Source* | *Address* |
|---|---|---|
| Adobe Photoshop 6.0 | Adobe Systems Inc. | www.adobe.com |
| AQM Advance 6 | Kinetic Imaging Ltd | www.kineticimaging.com |
| ImageJ | National Institutes of Health, USA | http://rsb.info.nih.gov/ij |
| LSM510 software | Carl Zeiss | www.zeiss.com |
| Mathmatica 5.0 | Wolfram Research | www.wolfram.com |
| MetaMorph 5.01 | Universal Imaging Systems | www.universal-imaging.com/ |
| Motion Analysis | Andor Technology | www.andor.com |

2.5.4: Random migration

BMMs were seeded onto 35-mm tissue culture plastic dishes (Nunc) at $2x10^5$ cells/well and filmed with timelapse microscopy. During timelapse microscopy experiments, cells were incubated in a humidified chamber at 10% $CO_2$. A stage heated to 37°C was used to control the temperature of the chamber. The chamber was set up on a Zeiss Axiovert 135 microscope and phase-contrast images were taken using a Hitachi Denshi KPM1E/K-S10 768 x 576 pixel, 8 bit CCD camera. A 10x Plan Neofluar N.A. 0.30 lens (Zeiss) was used for magnification. Image sets were collected using Kinetic Imaging Motion Analysis software with frames collected every 10 minutes for 8 hours.

2.5.5: Migration analysis

Cell migration was quantified by tracking the nucleus of the cell with Kinetic Imaging Motion Analysis software. Cells that left the field of view during the course of the timelapse movie or entered into mitosis were excluded from

analysis. Tracking of the cell nucleus gave details about the x and y co-ordinates of each cell in each frame and the displacement of the cell in pixels from frame to frame. These data were recorded and used to determine the speed and direction of the BMMs population. Software written for Mathematica 5.0 by G. Dunn (Zicha et al., 1997) was used to analyse the data.

2.5.6: Analysis of migration persistence

The persistence (P) of BMMs random migration was determined. This is determined by measuring the total distance migrated by a cell and its overall displacement from its starting position (Figure 5.3A). Using the Motion Analysis program, the displacement of the migrating cell was measured for each frame and saved as a Microsoft Excel compatible file (.xld). An Excel macro was developed within the laboratory by Dr. A. Wheeler which calculated the persistence for the population of cells based upon the following equation.

$$P_n = \frac{\text{Displacement of a cell (n) } (x_{ff},y_{ff}-x_{f1},y_{f1})}{\text{Total distance a cell (n) has moved } \{(\delta x,y_{f1})+(\delta x,y_{f2})+\ldots (\delta x,y_{f3})\}^n}$$

Where: n = an individual cell

$x_f,y_f$ = coordinates of a cell for a particular frame

$x_{ff}y_{ff}$ = final coordinates of a cell

$$P_{pop}= \frac{\Sigma\, Pn_{f1}+n_{f2}\ldots n_{ff}}{n_{pop}}$$

The Student's *t*-test was used to compare the persistence between different BMMs genotypes.

2.5.7: Spreading analysis

Glass-bottomed dishes (MatTek Corp.) were marked using a diamond tipped marker to allow easier focusing of the glass surface. 2 ml of BMMs growth medium was added to the dish and placed in a humidified chamber with 10% $CO_2$ at 37°C. A Nikon Eclipse TE 2000-E Microscope with a Hamamatsu Photonics ORCA-ER CCD camera and a 40x Nikon Plan Fluor 1.30 NA oil objective was used to focus on the surface of the glass bottomed dish. 1x $10^5$ cells in macrophage growth medium were added and image capture begun. Image sets were taken using the AQM Advance 6 software with frames every 10 seconds for 30 minutes and saved as Tiff single image files.

To analyse spreading between different populations, the number of lamellipodia and the size of the lamellipodia were measured. Using every 20$^{th}$ frame from the spreading time-lapse movies, the number of lamellipodia a cell extended was counted. To measure the lamellipodia, firstly, a line was drawn from the edge of the cell body to the edge of the lamellipodium (designated the width) and was measured by MetaMorph. Secondly, a line was drawn tracing the edge of the lamellipodium (designated the perimeter) and was measured by MetaMorph (Figure 2.2). The data obtained were exported to Microsoft Excel for statistical analysis. Student's *t*-test was used to investigate any statistical differences between populations.

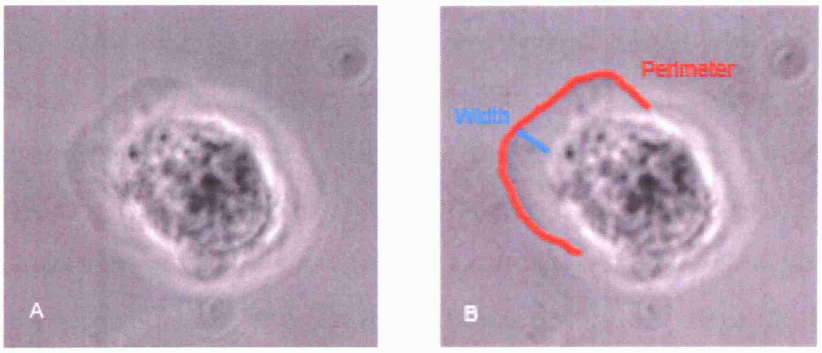



**Figure 2.2:** Quantification of lamellipodial size.

A) Phase contrast image of spreading WT BMMs.
B) Image showing the perimeter and width measurements to determine lamellipodial size.

2.5.8: Scratch assay analysis

For low magnification studies, BMMs were plated onto 6-cm bacteriological plastic dishes (Falcon) at 1x $10^6$ cells/well. BMMs were grown for 48-72 hours in a humidified chamber at 37°C and 10% $CO_2$ until a confluent layer of cells had formed. Cells were washed and growth medium was replaced before a sterile 200 μl pipette tip was used to produce a scratch through the cell layer. The dish was placed into the Zeiss Axiovert 135 microscope humidified chamber at 37°C and 10% $CO_2$ and images were collected as in random migration assays. Frames were taken every 10 minutes for up to 20 hours.

For higher resolution analysis, BMMs were seeded as before but onto a glass-bottomed dish and the Nikon ECLIPSE TE-2000-E microscope was used in conjunction with the Nikon 40x objective. The scratch was produced as before. Image sets were collected using the AQM advance 6 software with frames taken once a minute for 2 hours.

2.5.9: Dunn chamber chemotaxis assays

BMMs were seeded onto acid-washed 22 x 22-mm coverslips at 4x$10^4$ cells/well in growth medium and incubated in a humidified chamber at 37°C and 10% $CO_2$ for 6 hours. The coverslips were then incubated in macrophage starve medium overnight. The coverslips were mounted onto Dunn chemotaxis chambers (Wells et al., 2004) with recombinant murine CSF-1 (33 ng/ml) added as the chemoattractant (Figure 2.3). The Dunn chamber was sealed with wax made of lanolin, Vaseline and paraffin wax in a 1:1:1 ratio and placed into the microscope chamber heated to 37°C. Phase contrast images were collected every 10 minutes for 18 hours using the Kinetic Imaging Motion Analysis software. Cells were tracked using the Motion Analysis software and Mathematica 5.0 was used to analyse the tracking data.

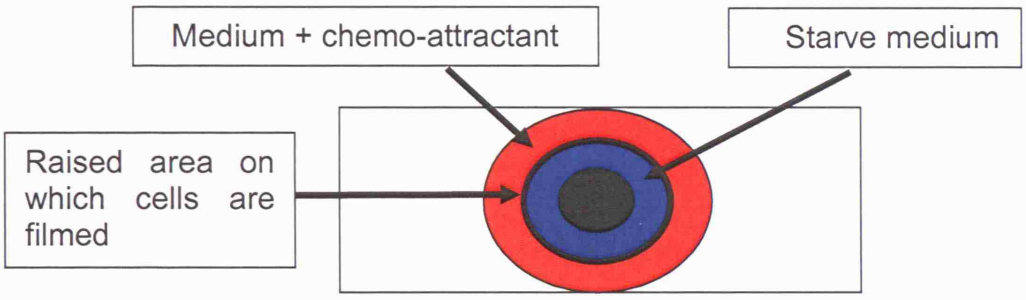



**Figure 2.3:** Diagram showing the layout of a Dunn chemotaxis chamber.

2.5. 10: Transwell chemotaxis assays

The upper chambers of 5 µm-pore polycarbonate Transwell filters were coated using macrophage starve medium for 30 minutes at 37°C. The starve medium from the upper well was removed and $1x10^5$ BMMs suspended in starve medium were added. Macrophage starve medium containing 33 ng/ml recombinant murine CSF-1 was added to the bottom chamber and was incubated at 37°C and 10% $CO_2$ for 24 hours. BMMs that did not migrate through the pores were removed from the upper chamber using a cotton bud and the Transwell filter was washed in PBS. Migrated BMMs were fixed and stained using the REASTAIN Quick-Diff kit (Reagena). The filter was removed, mounted onto a slide using mounting medium and covered with a coverslip. Using the Zeiss Axiophot microscope, 10 random frames were picked for each filter using a 100x Zeiss Plan-Neofluar 1.30 N.A oil objective and the cell number was determined. The data were analysed in Excel and the Student's *t*-test was used to investigate any significant differences.

2.5.11: Immunofluorescence

BMMs were seeded onto 13-mm diameter glass coverslips at $1x10^4$ cells/well and incubated in a humidified chamber at 37°C and 10% $CO_2$ for the length of time stated in the figure legends. The cells were fixed in 4% para-formaldehyde in PBS. Cells were permeabilised in 0.5% Triton X-100 in PBS for 5 minutes and then blocked with 20% goat serum in PBS for 30 minutes at RT. Primary antibodies were used as shown in Table 2.3 and were diluted

in 20% goat serum. Cells were incubated in primary antibody either at RT for 1 hour or at 4°C overnight. Excess primary antibody was removed through washing with PBS and the appropriate secondary antibodies diluted in PBS were added for 1 hour at RT. Rhodamine-conjugated phalloidin was added with the secondary antibodies to visualize F-actin. Coverslips were mounted onto slides using mounting medium and left to dry overnight at 4°C.

2.5.12: Confocal Microscopy

A Zeiss LSM510 confocal laser-scanning microscope, with a 40x / 1.30 N.A. plan neofluar objective and the LSM510 software were used to generate images of immunofluorescently stained cells. The settings for each fluorophore used in conjunction with the confocal microscope were:

| Fluorophore | Laser | Excitation $\lambda$ (nm) | Emission $\lambda$ (nm) | Filter used (nm) |
|---|---|---|---|---|
| FITC | Argon | 488 | 520 | Band pass 505-550 |
| TRITC/Rhodamine | Helium/Neon | 543 | 570/590 | Long pass 560 |
| Cy5 | Helium/Neon | 650 | 670 | Long pass 680 |

**Table 2.4:** Confocal microscope filter setup

Image files were collected as a matrix of 1024 x 1024 pixels that described the average of 8 frames scanned at 0.062 Hz. These files were then exported into 16 bit Tiff files which were used for image analysis.

2.5.13: Analysis of cell spread area and elongation ratio

To analyse the rate at which BMMs spread and elongate, cells were seeded onto 13-mm diameter coverslips at a density of 1x $10^4$ cells/well. BMMs were left to adhere for either 5, 15, 30 and 60 minutes or for 24 hours in a humidified chamber at 37°C and 10% $CO_2$ before washing, fixing,

permeablisation and staining for F-actin with rhodamine-conjugated phalloidin.

To analyse the spread area and elongation ratio of the BMMs at each time point, images were taken using the Zeiss LSM510 confocal microscope. Images were converted into 8 bit Tiff files and pre-processed using Adobe Photoshop 6.0 and ImageJ to remove fluorescently labelled cell debris. MetaMorph 5.01 was then used to quantitate the area and elongation ratio of the cells. Briefly; a median filter using a 3x3 kernel was used on the images to remove interference from background light. The image was then converted into a binary threshold image in which all pixels above a certain grey-scale level (when whole cell is highlighted) which were deemed of interest were assigned a value of 1. All other pixels are given a value of 0. Thus, the pixels assigned a value of 1 should correspond to the pixels of the spread cell. The integrated morphometry analysis function within MetaMorph was then used to determine the area of the cell, the longest chord through the cell and then the widest point perpendicular to this chord. Occasionally, background pixels were present in the analysis image. To prevent this, MetaMorph ignored all positive areas that consisted of fewer than 250 pixels (corresponding to 5 $\mu m^2$). The numerical data obtained in MetaMorph were exported to Microsoft Excel. The elongation of the cell (designated as the Aspect) was calculated by dividing the longest chord of each cell by the breadth at its widest point. Statistical analysis of spread area and elongation was carried out using Microsoft Excel and Student's *t*-test was used to compare differences between groups. Statistical significance was accepted for $p<0.05$. Data are presented as means ± s.e.m.

2.5.14: Analysis of membrane ruffling

To analyse membrane ruffling in macrophages, WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips at $1x10^4$ cells/well. BMMs were starved of CSF-1 for 16 hours and then stimulated with 33 ng/ml CSF-1. BMMs were fixed and stained with rhodamine-conjugated phalloidin before confocal images of the

basal and apical planes of the cell were acquired. Macrophages were scored for the presence of central or peripheral ruffles or a total absence of them.

### 2.5.15: Adhesion assay

WT and PAK1$^{-/-}$ BMMs were plated onto tissue culture plastic for between 5 and 60 minutes. Non-adherent cells were washed off with PBS before addition of growth medium containing 25 $\mu$M MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 2 hours. Addition of MTT to the cell medium results in its absorption by the BMMs. Once in the BMMs, mitochondrial dehydrogenases from viable cells cleave the tetrazolium rings of the yellow MTT and form dark blue formazan crystals. After 2 hours, the BMMs were lysed with 5% SDS and the OD (570 nm) of the blue formazan crystals was determined.

## 2.6: Flow cytometry:

### 2.6.1: Buffers

| | |
|---|---|
| FACS buffer | 5% endotoxin-free BSA (w/v) in PBS |

### 2.6.2: Equipment

BD FACS Canto flow cytometer

### 2.6.3: Software

| *Programme* | *Manufacturer* | *Address* |
|---|---|---|
| FACS Diva | Becton Dickinson | www.bd.com |

2.6.4: Antibodies used for flow cytometry

| Flow Cytometry | | | | |
|---|---|---|---|---|
| Antibody | Reactivity | Dilution | Source | Address |
| FITC-F4/80 | Rat | 1:100 | Serotec | www.serotec.co.uk |
| FITC-IgG2b neg. control | Rat | 1:100 | Serotec | www.serotec.co.uk |
| FITC-Integrin $\beta$1 | Rat | 1/100 | BD Pharmingen | www.bdbiosciences.com |
| PE-Integrin $\beta$2 | Mouse | 1/100 | BD Pharmingen | www.bdbiosciences.com |

**Table 2.6:** Conditions of antibodies used for flow cytometry.

2.6.5: Flow cytometry protocol

BMMs were grown to approximately 80% confluence in 6-cm bacteriological plastic dishes and passaged in versene. Cells were collected by centrifugation at 1100 rpm (ALC PK130R) for 5 minutes, resuspended in FACS buffer and aliquoted into 1.5 ml eppendorf tubes at a concentration of $5x10^5$ cells/ml. 1 µg/ml of antibody was added to the eppendorf tubes for 30 minutes on ice and the cells were then centrifuged and washed twice in FACS buffer. 0.4% para-formaldehyde was added for 10 minutes to fix the cells and the cell surface fluorescence was measured using a flow cytometer. The data were analysed using the FACS Diva software.

# Chapter 3: PAK regulation in bone marrow-derived macrophages.

## 3.1: Introduction

CSF-1 is a macrophage differentiation, proliferation and survival factor which is secreted by a number of cell types (Stanley et al., 1997). Previous research has shown that macrophages required Rho, Rac and Cdc42 signalling for directional migration towards CSF-1 (Allen et al., 1998) and that CSF-1 is capable of activating Rac (Grill and Schrader, 2002). Stimulation of BMMs with CSF-1 stimulates the MAP kinase pathway and hence, upregulates transcription of a number of gene targets (Hamilton, 1997). This suggests that CSF-1 stimulation of macrophages may play an important role in the activation of the Rho GTPases and its downstream targets such as the PAK family. This makes macrophages an interesting model for the study of PAK regulation and signalling.

The cytokine TNF$\alpha$ also stimulates macrophages *in vivo*, and was used to investigate whether pro-inflammatory signalling to macrophages affected the activation or regulation of PAK. TNF$\alpha$ is secreted in areas of infection and cell damage to promote an immune response. Macrophages play a vital role in the TNF$\alpha$ induced immune response and TNF$\alpha$ has been shown to promote a number of intracellular signalling pathways upon stimulation (see review (MacEwan, 2002)).

The PAKs have had a number of regulatory mechanisms identified. The traditional view of PAK activation involves the binding of the Rho GTPases Rac or Cdc42 to the PAK CRIB domain which disrupts an auto-inhibitory dimer (Lei et al., 2000; Parrini et al., 2002) allowing the phosphorylation (possibly auto-phosphorylation or by PDK1 (Benner et al., 1995; King et al., 2000; Yu et al., 1998)) of residues within the PAK kinase and auto-inhibitory domains. This leads to conformational changes forming an active kinase domain (Lei et al., 2005). PAK signalling is also regulated by the binding of adaptor proteins and its subsequent targeting within the cell (Brown et al.,

2002; Li et al., 2001; Zhao et al., 2000) and by phosphorylation of a number of residues throughout the protein (Manser et al., 1997).

To determine whether the stimulation of macrophages with CSF-1 or TNF$\alpha$ led to the activation and regulation of the PAKs, BMMs were generated from WT C57B6 mice as has been described previously (Vairo and Hamilton, 1985).

## Results

### 3.2: BMMs express PAKs 1, 2 and 3

Before PAK regulation could be investigated, it was first necessary to establish the macrophage PAK expression profile. Previous research has shown that whilst PAK2 is expressed ubiquitously, the other PAK family members have distinct expression profiles (Kumar and Vadlamudi, 2002).

RT-PCR showed that PAK1, PAK2 and PAK3 mRNA was present in BMMs but not PAK4, PAK5 or PAK6 (Figure 3.1A) and was confirmed using western blotting (Figure 3.1B). It was possible to identify the presence of PAKs 1, 2 and 3 in BMM cell lysate although PAK3 was generally very faint and difficult to detect suggesting low expression levels in BMMs. Antibodies raised against PAK4 and PAK5 gave no signal (data not shown). The PAK1 specific antibody recognised PAK1 as a doublet on western blots. This has been seen previously in the literature (Misra et al., 2005; Singh et al., 2005) although little is mentioned of it. Singh *et al.* suggest that the doublet seen for soluble PAK1 is a result of different PAK1 phosphorylated and active forms. However, Calf Intestine Alkaline Phosphatase (CIP) removal of phosphorylation sites on PAK1 did not affect the doublet (data not shown) suggesting that the doublet is not a result of PAK1 phosphorylation and may be due to a different mechanism of protein modification.

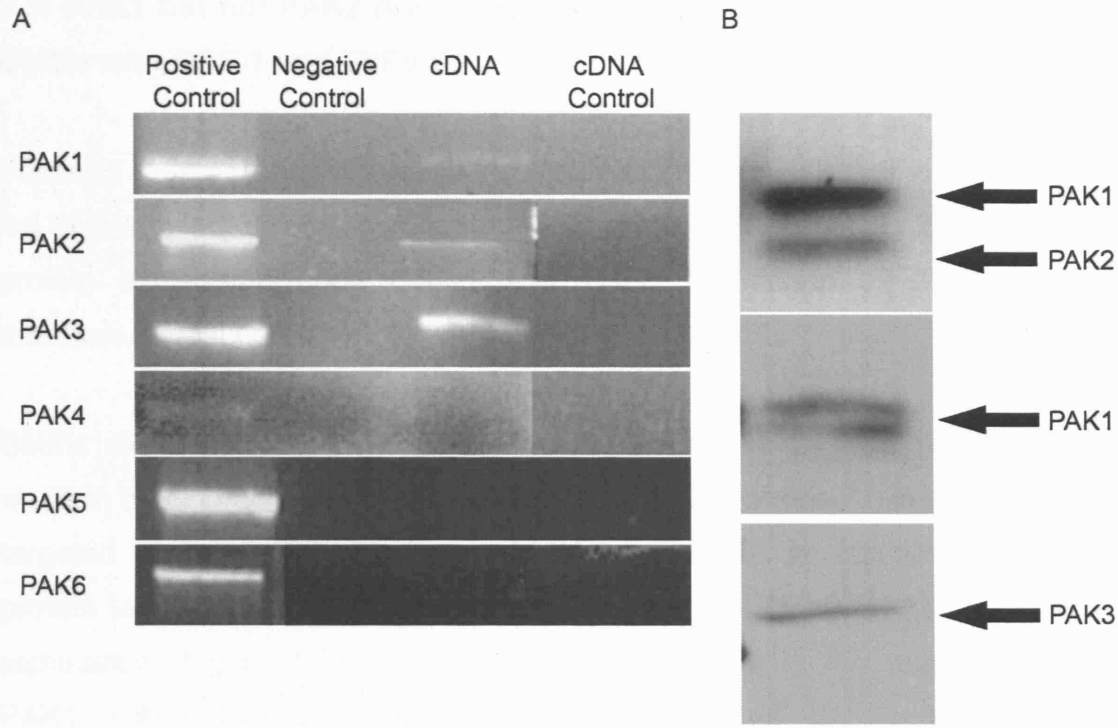



**Figure 3.1:** WT BMMs express PAK1, 2 and 3.

(A) Total cellular RNA was isolated from BMMs cultured in growth medium and cDNA was performed. The PAK expression profile in WT BMMs was analysed by reverse transcription PCR (RT-PCR). Plasmids encoding the mouse cDNA for Myc-PAK1, Myc-PAK2, HA-PAK3, HA-PAK4, Myc-PAK5 and Flag-PAK6 were used as positive controls. Negative controls contained no cDNA and the cDNA control was prepared in the absence of reverse transcriptase. B) Cell lysate from BMMs in growth medium was made and probed via western blotting with the antibodies C-19 ($\alpha$-PAK1, 2 and 3), $\alpha$-PAK1 and $\alpha$-PAK3. Figures shown are representative of three separate experiments. RT-PCR blots that have been pasted together are a result of lanes being separated. RT-PCR experiments were run on the same day and on the same gel.

### 3.3: PAK1 but not PAK2 protein levels are elevated upon stimulation of BMMs with CSF-1 and TNF*α*

Previous research has shown that phorbol ester stimulation of HepG2 cells led to a transient down-regulation of PAK1 protein (Gujdar et al., 2003). PAK protein expression upon CSF-1 and TNF*α* stimulation of BMMs was, therefore, investigated.

BMMs were stimulated with CSF-1 or TNF*α* for 30 to 360 minutes and western blotting analysis on the cell lysates was performed. Using antibodies targeted against either the group A PAKs or PAK1 specifically, the total protein levels were investigated. CSF-1 stimulation (Figure 3.2A) and TNF*α* stimulation (Figure 3.2B) both induced an increase in the total levels of PAK1. PAK2 was unchanged in CSF-1 stimulated BMMs and largely unchanged in TNF*α* stimulated BMMs although a small increase may be visible at 4 hours after stimulation. However, the increase in PAK2 is considerably smaller than that seen in PAK1 protein levels suggesting CSF-1 and TNF*α* are principally capable of regulatory control over PAK1.

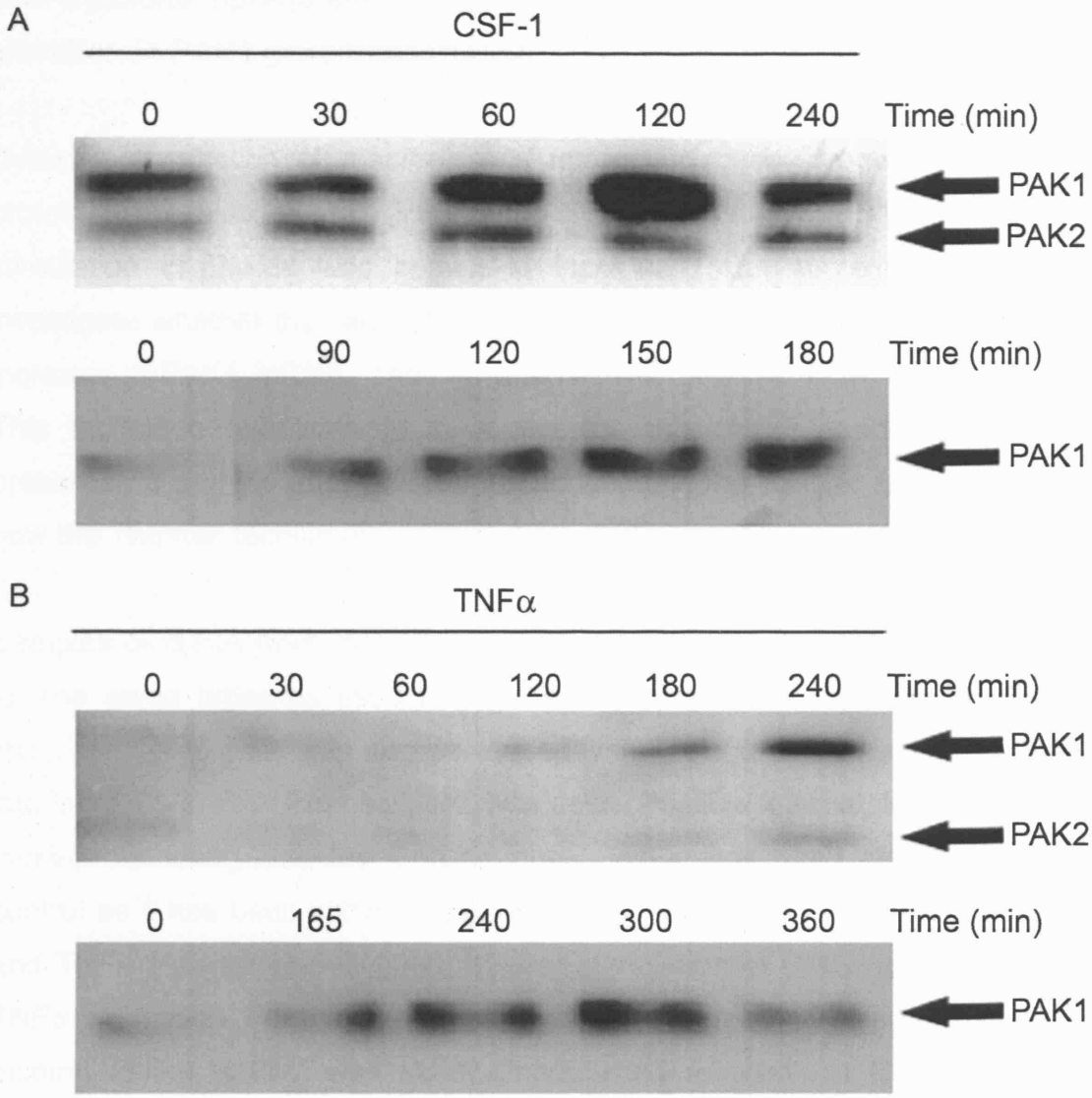



**Figure 3.2:** CSF-1 and TNF$\alpha$ increase PAK1 protein expression in WT BMMs

BMMs were starved of CSF-1 for 16 hours and then stimulated with either A) 33 ng/ml CSF-1 or B) 10 ng/ml TNF$\alpha$. Changes in PAK1 expression were analysed by western blotting with antibodies C-19 (PAK1, 2 and 3) and $\alpha$-PAK1. Figures shown are representative of three separate experiments.

### 3.4: Cytokine upregulation of PAK1 protein levels is not due to an elevation in PAK1 gene transcription

Cytokine stimulation of macrophages upregulates gene transcription and protein expression *in vivo* (Pixley and Stanley, 2004). CSF-1 and TNF$\alpha$ stimulation of BMMs was shown to increase PAK1 protein levels. To investigate whether the rise in PAK1 protein levels observed was due to an increase in PAK1 mRNA, Taqman quantitative real-time PCR was utilised. This technique quantitatively measures the amount of a specific cDNA present in a sample after reverse transcription of the mRNA. A diagram of how the Taqman technique works is shown in Figure 2.1.

Samples of cDNA were made from BMMs stimulated with CSF-1 and TNF$\alpha$ for the same times as used in the western blotting experiments. A 24hr stimulation time point was also performed with TNF$\alpha$ to determine whether a late increase in PAK1 transcription was seen. Positive internal controls were carried out along side the PAK1 probes. TNF$\alpha$ was used as an internal control as it has been shown that both CSF-1 (Branch and Guilbert, 1996b) and TNF$\alpha$ (Branch and Guilbert, 1996a) stimulation of BMMs can result in TNF$\alpha$ production. For the 24 hour stimulation experiment, Siglec1 (sialic acid binding Ig-like lectins) was used. Unpublished research at CellTech had shown that TNF$\alpha$ stimulation of monocytes led to an increase in Siglec-1 mRNA levels (Dr J. Munday, personal communication).The Siglec family are mainly expressed by haematopoietic cells and have been shown to help mediate specific cell: cell interactions (Crocker and Varki, 2001) as well as the immune systems inflammatory response including production of pro-inflammatory cytokines (Lajaunias et al., 2005) and enhancing neutrophil oxidative burst (Erickson-Miller et al., 2003).

Taqman analysis of PAK1 cDNA levels after CSF-1 (Figure 3.3A) and TNF$\alpha$ stimulation (Figure 3.3B and C), showed only a slight increase over starved levels, probably constituting a return to normal growth levels rather than a stimulation induced increase. No further increases are seen over time following stimulation. Siglec-1 and TNF$\alpha$ mRNAs both increased after

stimulation showing that stimulation of the BMMs was successful. This shows that PAK1 upregulation upon cytokine stimulation is not due to an increase in gene transcription but TNF*α* production after CSF-1 or TNF*α* stimulation is.

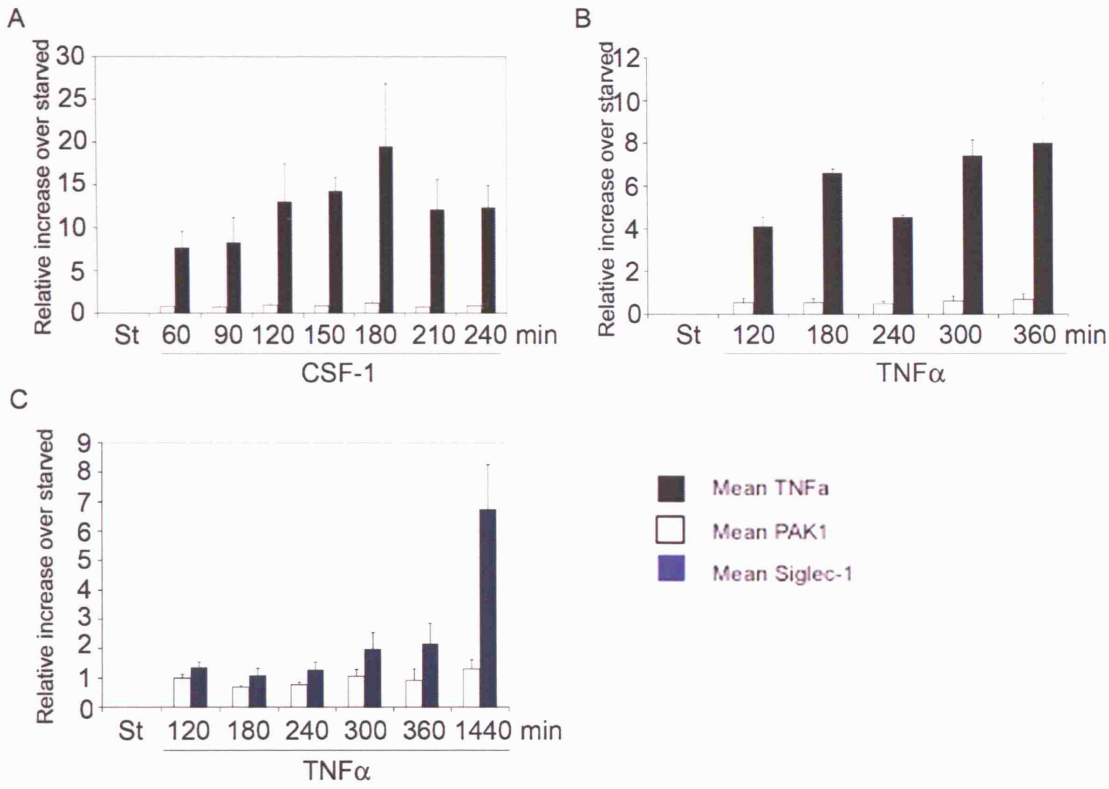



**Figure 3.3:** CSF-1 and TNF*α* stimulation does not alter PAK1 mRNA levels

BMMs were starved of CSF-1 for 16 hours and then stimulated with CSF-1 or TNF*α*. RNA was isolated and reverse transcribed into cDNA. Taqman quantitative RT-PCR analysis of PAK1 (white bars) and TNF*α* mRNA (black bars) levels after stimulation with (A) 33 ng/ml CSF-1 and (B) 10 ng/ml TNF*α*. C) Taqman analysis of PAK1 (w.b) and Siglec-1 (blue bars) mRNA levels after 10 ng/ml TNF*α* stimulation. All results were equalised to an internal loading control probe targeted against 18s rRNA and show the mean +/- s.e.m of three separate experiments.

### 3.5: PAK1 is a stable protein that is not targeted to the proteasome

Increased PAK1 protein levels upon CSF-1 stimulation are not a consequence of more PAK1 mRNA being transcribed. However, protein expression can also be regulated by an increase in mRNA translation or changes to the rate of protein degradation.

Ubiquitin is a highly evolutionarily conserved, 76 amino acid protein that is involved in protein targeting. Covalently linking ubiquitin to a number of proteins intracellularly results in either a modification of the proteins activity or its degradation via the proteasome. Current dogma suggests that binding of a single ubiquitin affects the target proteins activity or location whereas binding of many ubiquitins (a polyubiquitin chain) results in targeting to the proteasome (Wilkinson, 2000). It has been shown that caspase-activated PAK2 is regulated via polyubiquitination targeting to the proteasome (Jakobi et al., 2003) raising the possibility that PAK1 is also regulated in this manner.

The stability of PAK1 protein and whether it is targeted to the proteasome for degradation was investigated. Use of the protein translation inhibitor, cycloheximide, can give an indication of the protein half-life. Use of cycloheximide on BMMs (Figure 3.4A) showed that PAK1 levels did not appreciably decrease over a four hour period whilst cyclin D1 was almost entirely absent after two hours inhibition. Cyclin D1 was used as a degradation control due to its rapid turnover with a half-life of just 27 minutes in macrophages. UV irradiation of the Bac1.2F5 macrophage cell lines and BMMs showed that cyclin D1 mRNA was cleared within a minute and cyclin D1 protein was targeted to the proteasome (Miyakawa and Matsushime, 2001). Therefore, in comparison to cyclin D1, PAK1 protein is stable and has a low turnover rate in BMMs.

A number of intracellular proteins are degraded via the proteasome as part of their regulation, for example cyclin D1. To investigate whether the PAK1 protein is targeted through this pathway, the proteasome inhibitors ALLnL (N-acetyl leucyl-leucyl norleucinal) and Lactacystin were utilised. Using western

blotting, levels of PAK1 were visualised over a time course of inhibition with ALLnL (data not shown) and after 24 hours exposure to Lactacystin (Figure 3.4B). The western blots showed that the levels of PAK1 did not change with proteasome inhibition suggesting that PAK1 is not targeted to the proteasome.

Further evidence to investigate whether PAK1 is degraded by the proteasome could be determined by investigating potential PAK1 ubiquitination after stimulation.

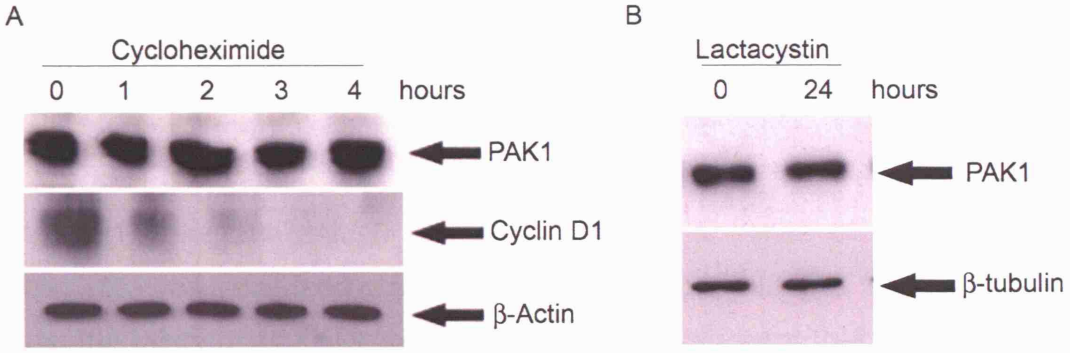



**Figure 3.4:** PAK1 is a stable protein but is not regulated by targeting to the proteasome.

BMMs were cultured in growth medium before inhibitor addition. PAK1 protein stability was investigated by A) western blot analysis of PAK1 and cyclin D1 protein levels after protein translation inhibition using 10 mg/ml cycloheximide. To determine whether PAK1 was regulated via the proteasome, B) western blot analysis of PAK1 protein levels after proteasome inhibition using 10 mg/ml Lactacystin was performed. Western blots were reprobed for $\beta$-actin and $\beta$-tubulin levels as a loading control. Blots shown are representative of at least two separate experiments.

### 3.6: Cytokine stimulation of PAK1 upregulation does not require new protein translation

BMM stimulation with CSF-1 or TNF$\alpha$ led to an increase in PAK1 protein levels which is not due to an increase in mRNA levels. Another possible explanation for the increase seen in PAK1 is, rather than an increase in PAK1 mRNA levels, there is an increase in the translation of pre-existing mRNA.

To investigate this possibility, BMMs were stimulated with CSF-1 in the presence of the protein translation inhibitor cycloheximide. Western blotting (Figure 3.5A) showed that despite protein translation inhibition, the increase in PAK1 protein levels associated with CSF-1 stimulation was observed. To check that the inhibition of protein translation was effective, the levels of cyclin D1 were analysed (Figure 3.5B). In the absence of cycloheximide, cyclin D1 was produced upon CSF-1 stimulation, but this was prevented when the inhibitor was also added. These results suggest that the increase in PAK1 levels upon cytokine stimulation of BMMs is not due to a change in gene transcription or protein translation.

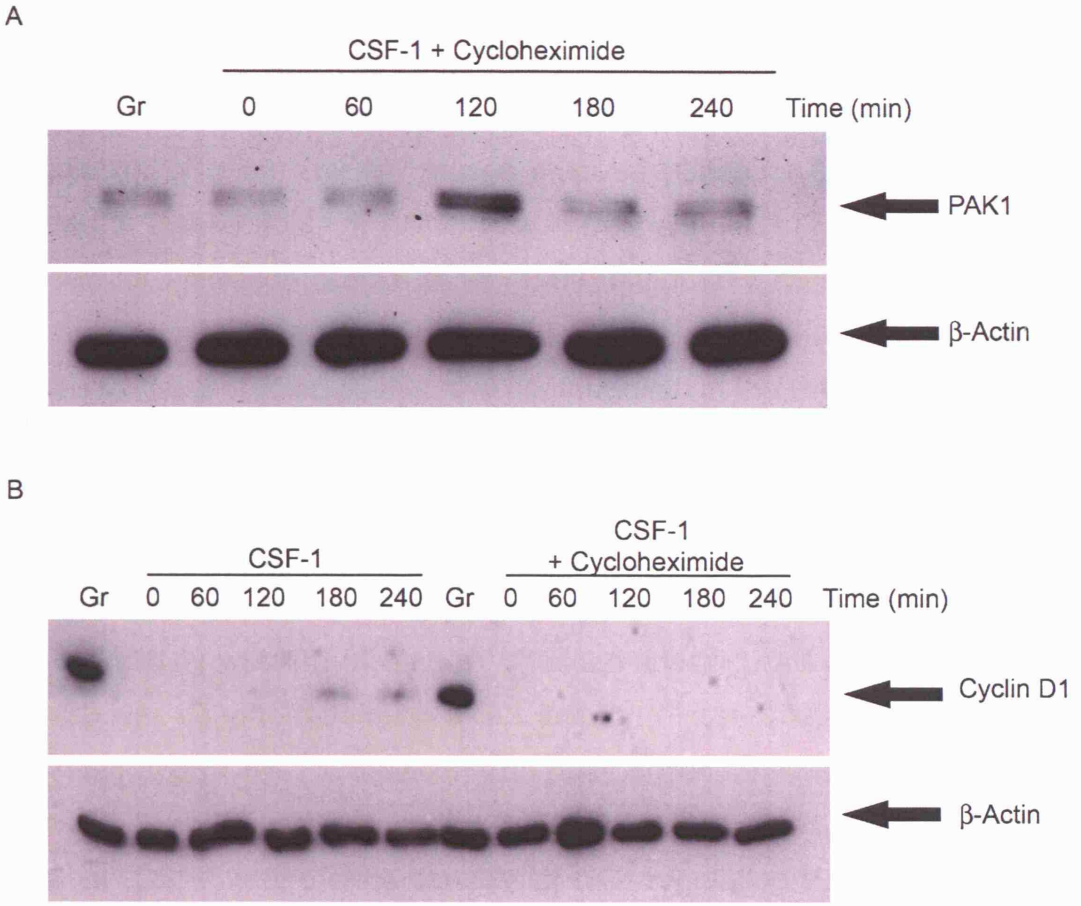



**Figure 3.5:** PAK1 protein levels increase upon stimulation independently of new protein synthesis.

BMMs were either maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. A) Western blot showing protein synthesis inhibition by cycloheximide (10 mg/ml) does not stop the increase in PAK1 levels seen after CSF-1 (33 ng/ml) stimulation. The PAK1-specific antibody was used to identify PAK1 levels. To check that protein synthesis inhibition was successful (B), WT BMMs were incubated in either CSF-1 or CSF-1 plus cycloheximide. The recovery of cyclin D1 levels in BMMs stimulated without cycloheximide confirmed the inhibition of protein synthesis.

### 3.7: CSF-1 stimulation of BMMs leads to phosphorylation and activation of PAK1

Regulation of a number of kinases involves phosphorylation of a residue within the kinase domain. Within PAK1, threonine 423 (Thr423) is a critical residue for activation (Zenke et al., 1999) although a number of other sites are also phosphorylated (Manser et al., 1997). Phosphorylation at Thr423 promotes the formation of an active conformation in the PAK1 kinase domain leading to initiation of a number of signalling pathways.

To investigate whether CSF-1 induced PAK1 activation as well as upregulated its protein levels, phosphorylation of Thr423 in PAK1 was determined by western blotting. A phospho-specific antibody against PAK1 Thr423 was used to determine PAK1 activity (Figure 3.7). PAK1 was rapidly activated upon CSF-1 stimulation with peak phosphorylation levels observed between 2 and 5 minutes before they return to a lower level again by 10 minutes. CSF-1 is therefore capable of both regulating PAK1 protein levels and promoting PAK1 kinase activity.

It is also possible that CSF-1 stimulation of BMMs induces activation of PAK2 and PAK3. The antibody is stated to be a PAK1 Thr423, PAK2 Thr402 specific antibody and should recognise activated species of both PAK proteins although it may also cross-react with active PAK3. The antibody was raised with a synthetic phospho-peptide corresponding to the area of PAK1 phosphorylation. However, sequence alignment of human PAK1, 2 and 3 shows very high levels of sequence conservation in the kinase domain around this residue (see review (Bokoch, 2003)). The mouse sequences for PAK1, 2 and 3 were aligned using the SIM alignment tool at ExPASy to determine the sequence homology around the phosphorylated threonine residue (Figure 3.6). The 20 amino acid sequence around the PAK1 Thr423 site shows no changes in PAK2 and PAK3 indicating that this region is very highly conserved and likely to be recognised by the antibody.

Stimulation of PAK3 is hard to establish. Its similarity in size to PAK1 and the low levels at which it appears to be expressed in BMMs make determining its activation difficult. However, with its expression being low, it seems unlikely that it will have a major role in the downstream signalling of CSF-1. PAK2 activation has to be differentiated from PAK1 activation by the molecular weight (Mw) of the bands visualised. The antibody, however, frequently does not produce discreet bands when used on stimulated macrophages but instead recognises a number of bands indicating the presence of numerous phosphorylated species. The Mw of the bands are around the size of PAK1 (65 kDa), however, suggesting the phosphorylated protein observed is PAK1.

mPAK1 GFCAQITPEQSKRSTMVGTPY
mPAK2 GFCAQITPEQSKRSTMVGTPY
mPAK3 GFCAQITPEQSKRSTMVGTPY

**Figure 3.6:** The PAK kinase domain has 100% homology around the threonine residue critical for activation.

The amino acid sequence for mouse PAK1, PAK2 and PAK3 were obtained from the Entrez Protein website and aligned in the ExPASy SIM alignment tool. The 20 amino acids surrounding the critical threonine residue (highlighted in red PAK1= Thr423, PAK2= Thr402, PAK3= Thr421) are identical in each of the PAK proteins.

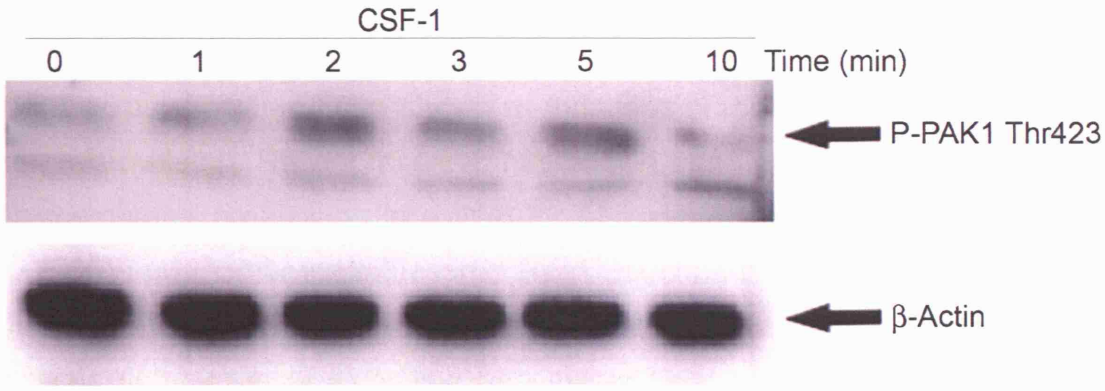



**Figure 3.7:** CSF-1 stimulation induces PAK1 phosphorylation in WT BMMs

BMMs were cultured in growth medium and then starved of CSF-1 for 16 hours. BMMs were then stimulated for 1-10 minutes with 33 ng/ml CSF-1. Cell lysates were resolved by SDS-PAGE, western blotted and probed with the phosphorylation-specific $\alpha$-PAK1/2 Thr423 antibody. The western blot was reprobed for total $\beta$-actin as a loading control. Western blots are representative of three separate experiments.

### 3.8: CSF-1 stimulation of BMMs induces membrane ruffling

CSF-1 stimulation has been shown to induce a number of changes to the actin cytoskeleton. Recent research has shown that CSF-1 induces F-actin-rich membrane protrusions in a WAVE2-Abi1 dependent manner (Kheir et al., 2005) and membrane ruffles in a Rac1 dependent manner (Wells et al., 2004). Activation of the CSF-1 receptor is also believed to form multimeric cytosolic complexes of signalling and cytoskeletal proteins (Yeung et al., 1998) suggesting that CSF-1 signalling plays a crucial role in cytoskeletal reorganisation.

The effect of CSF-1 stimulation on the actin cytoskeleton of BMMs was investigated at time points that correlated with PAK1 activity and protein upregulation. BMMs were starved of CSF-1 for 16 hours before stimulation with CSF-1 for between 2 and 120 mins (Figure 3.8). Images of both the basal and apical cell surface were taken to identify evidence for actin reorganisation into apical membrane ruffles. CSF-1 stimulation induced membrane ruffling within 2 minutes as previously reported (Wells et al., 2004), which continued for the period identified as having PAK1 activated. By 60 minutes after addition of the stimulus, ruffling was greatly reduced. However, 120 minutes after CSF-1 addition, cell ruffling on the apical surface was more prevalent. This increase in ruffling coincided with the rise observed in PAK1 protein levels (Figure 3.2).

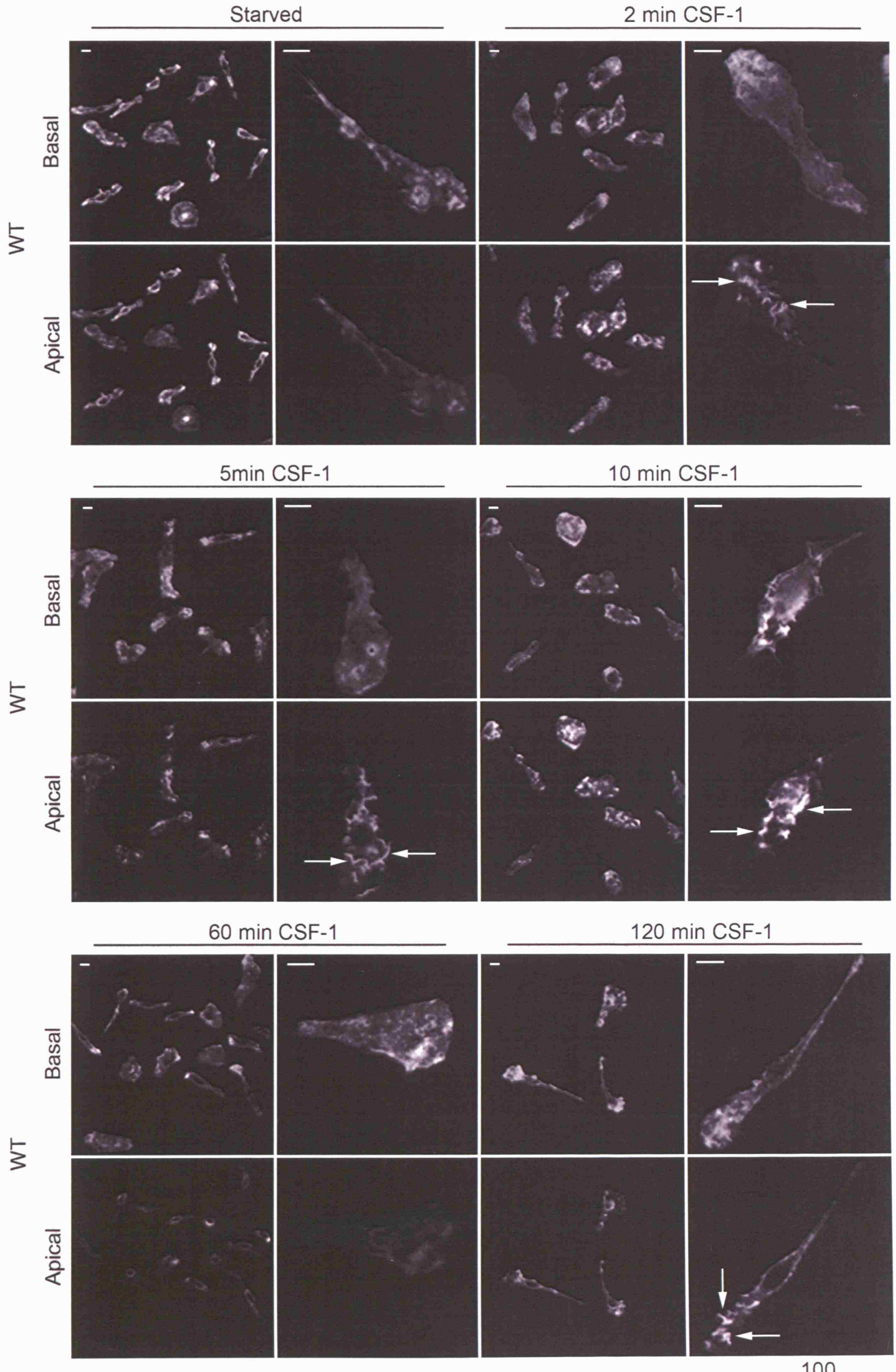

Starved
2 min CSF-1
5min CSF-1
10 min CSF-1
60 min CSF-1
120 min CSF-1
WT
Basal
Apical

**Figure 3.8:** WT BMMs produce membrane ruffles in response to CSF-1 stimulation.

BMMs were grown in growth medium for 24 hours on glass coverslips and then starved of CSF-1 for 16 hours. BMMs were stimulated with 33 ng/ml CSF-1 and confocal microscope images showing apical membrane ruffling were taken. Cells were stained for F-actin using TRITC-phalloidin. Bars on images represent 10 $\mu$m and arrows indicate membrane ruffles. Images are representative of three separate experiments.

### 3.9: Increased PAK1 levels do not coincide with an increase in PAK1 activity.

It was shown that CSF-1 stimulation of BMMs lead to an initial increase in phosphorylated PAK1 (Figure 3.7) and a later increase in total PAK1 protein levels (Figure 3.2). These changes in PAK1 were seen to coincide with membrane ruffling in BMMs (Figure 3.8) raising the possibility that PAK1 signalling is involved in this process.

CSF-1 stimulation of BMMs was shown to induce membrane ruffling in a Rac1 dependent manner (Wells et al., 2004) suggesting that Rac1 may activate PAK1 signalling pathways involved in membrane ruffling. The increase in PAK1 activation upon CSF-1 stimulation corresponding to ruffling supports this possibility. However, membrane ruffling was also observed in later time points when total PAK1 protein levels were elevated.

To determine whether an increase in PAK1 activity is observed at the same time as elevated PAK1 levels, western blotting was used (Figure 3.9). CSF-1 starved WT BMMs were stimulated with CSF-1 for between 5 and 210 mins. Western blotting with a PAK1 specific antibody showed an increase in PAK1 levels from 1 hour after stimulation which peaked at 2 hours and reduced afterwards. Western blotting with an antibody against phospho-PAK1/2 showed enhanced levels of active PAK1 between 5 and 10 mins and had reduced by 90 minutes. Over the period where total PAK1 levels were

elevated, no change in phosphorylated PAK1 was observed showing that increased PAK1 levels do not produce an increase in active PAK1. This indicates that either raised PAK1 levels are not involved in the production of membrane ruffles at late time points or that PAK1 is acting as a scaffold protein rather than a kinase. The blot was reprobed and it was possible to visualise PAK3 with a PAK3 specific antibody. Total PAK3 levels showed no changes over the course of CSF-1 stimulation and acted as a loading control for PAK1.

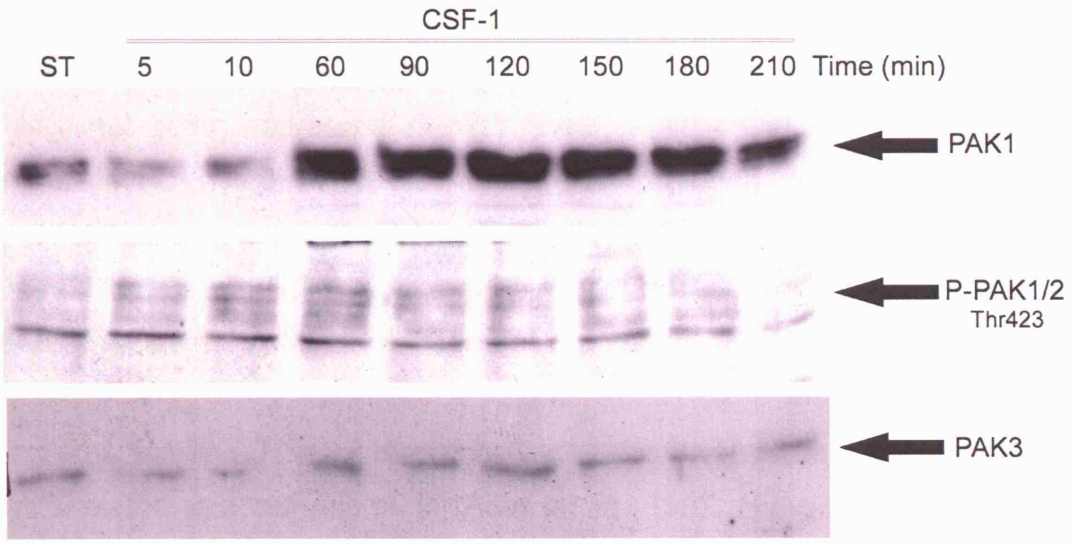



**Figure 3.9:** Increased PAK1 protein levels do not correspond with increased PAK phosphorylation.

WT BMMs were cultured in growth medium and then starved of CSF-1 for 16 hours. BMMs were stimulated with 33 ng/ml CSF-1 for between 5 and 210 minutes before cells were lysed. Western blotting was used to determine total PAK1 and phosphorylated PAK levels after CSF-1 stimulation. PAK3 levels are shown as a loading control and highlights that no changes in PAK3 levels are observed upon CSF-1 stimulation. Western blots are representative of two separate experiments

**3.10: Conclusions and discussion**

To study the role of PAKs in BMMs, it was first necessary to determine which PAK family members were expressed. Previous research using the macrophage cell line Raw 264.7, had shown the expression of PAK1 (Hullinger et al., 2001) but an exact PAK expression profile in macrophages was unknown. RT-PCR and western blotting showed that BMMs expressed PAK1 and PAK2 and low levels of PAK3. It was possible to ascertain that PAK3 was expressed at low levels due to the difficulty with which it was viewed on western blots with a PAK3 specific antibody. Further evidence for PAK3 not being expressed at high levels is shown in the $PAK1^{-/-}$ BMMs (Figure 4.1). Loss of PAK1 from the system removes the problem of PAK1 and PAK3 being too similar in Mw to distinguish between on a western blot. Use of the C19 anti-PAK1, 2 and 3 antibody showed no PAK3 band when PAK1 is absent. PAK4, 5 and 6 are not expressed in BMMs.

The Rho family GTPases Rac and Cdc42 are involved in regulation of the cytoskeleton and cell migration (Allen et al., 1997; Ridley and Hall, 1992). CSF-1 acts through Rac and Cdc42 to induce actin reorganisation (Allen et al., 1997) suggesting that macrophage responses to CSF-1 could involve PAK. CSF-1 stimulation of BMMs produced an increase in PAK1 but not PAK2 protein levels. Maximum PAK1 levels were observed between 120 and 180 minutes before returning to a basal level. An increase in PAK1 protein levels after CSF-1 stimulation suggests that PAK1 may play a critical role in CSF-1 signalling.

To determine whether this effect could be mimicked by another cytokine, BMMs were stimulated with TNF$\alpha$. TNF$\alpha$ stimulation of BMMs led to a similar increase in PAK1 protein levels but no or little change in PAK2. However, TNF$\alpha$ stimulation promoted PAK1 levels at a later time point with the peak in PAK1 being observed at 5 hours after stimulation.

TNF$\alpha$ is a pro-inflammatory cytokine that has profound effects upon macrophage function. Stimulation with TNF$\alpha$ leads to the production and

secretion of numerous other pro-inflammatory cytokines including TNF$\alpha$ itself (see review (Vassalli, 1992)). It has also been reported that CSF-1 stimulation of macrophages promotes the secretion of TNF$\alpha$ (Warren and Ralph, 1986) whilst TNF$\alpha$, but not TNF$\beta$, can induce macrophage CSF-1 secretion (Oster et al., 1987). Further evidence for a link between CSF-1 and TNF$\alpha$ is shown by studies that report Rac activation upon TNF$\alpha$ stimulation in the fibrosarcoma L929 cell line (Nosaka et al., 2001) and that TNF$\alpha$ is capable of inhibiting CSF-1 induced filopodia formation in various macrophage cell lines (Peppelenbosch et al., 1999). There is, therefore, a body of research that links CSF-1 and TNF$\alpha$ signalling making it unclear whether the observed increase in PAK1 was a direct consequence of TNF$\alpha$ stimulation or is an indirect effect of TNF$\alpha$ promoting the secretion of CSF-1 and hence, CSF-1 stimulation of the BMMs. Further studies are required to resolve this although the secretion of CSF-1 could explain the time delay seen in the TNF$\alpha$-induced increase in PAK1 protein.

Previous research has provided a precedent for PAK regulation through control of protein levels. Phorbol ester stimulation, as well as activating PAK, was shown to induce a transient decrease in PAK1 protein levels (Gujdar et al., 2003) although no stimulation-induced increase in PAK1 levels has been reported. CSF-1 and TNF$\alpha$, however, do signal to a number of pathways involved in transcription raising the possibility that stimulation may induce PAK1 transcription. CSF-1 stimulation induced the activation of STAT1 and STAT3 transcription factors in BMMs (Novak et al., 1995) and the formation of STAT5-containing DNA binding complexes in the Bac1.2F5 macrophage cell line (Novak et al., 1996). The Signal Transducer and Activator of Transcription (STAT) proteins are signal-dependent transcription factors that induce the transcription of a number of proteins involved in proliferation, differentiation, apoptosis, inflammation and the immune response (see reviews (Clevenger, 2004; Levy and Darnell, 2002)). The MAPK pathway has been reported to be another downstream target of CSF-1 signalling (Hamilton, 1997), possibly through PAK1 signalling (Bagrodia et al., 1995; Frost et al., 1996; Zhang et al., 1995) so CSF-1 can also regulate transcription via the MAPK pathway. The MAPK pathway activates a number

of transcription factors including Fos and Jun (see reviews (Seger and Krebs, 1995; Treisman, 1996)) which can regulate processes such as proliferation and apoptosis (see review (Shaulian and Karin, 2002)). These signalling pathways raise the possibility that CSF-1 stimulation of PAK1 protein levels may be through an induction of PAK1 transcription. At present, the method of PAK1 transcriptional regulation has not been established so it is unclear whether CSF-1 may regulate the transcription factor responsible for PAK1 induction.

Despite PAK1 transcriptional regulation not being reported yet, it is still possible to investigate increases in PAK1 transcription. Potential increases in PAK1 mRNA levels were investigated after CSF-1 and TNF$\alpha$ stimulation using Taqman RT-PCR. Stimulation of BMMs with CSF-1 or TNF$\alpha$ did not induce an increase in PAK1 gene transcription but did show increases in the positive controls. Therefore, the increase in PAK1 protein levels seen after BMMs stimulation is not due to an increase in gene transcription as would probably be expected. As CSF-1 upregulation of PAK1 is not due to an increase in transcription, regulation must be via another method. Other possible methods in which CSF-1 regulate PAK1 include changes to the proteins rate of degradation and an increase in mRNA translation rather than an increase in mRNA levels.

To investigate whether CSF-1 stimulation induced a change in PAK1 stability, cycloheximide inhibition of protein translation was utilised. Cycloheximide is an antibiotic that inhibits eukaryotic protein synthesis by blocking the translation of mRNA on cytosolic 80s ribosomes. Use of cycloheximide on BMMs showed no change in PAK1 protein levels after four hours whereas, cyclin D1 was completely absent after 3 hours. However, addition of cycloheximide is toxic to the BMMs and resulted in cell death after 4 hrs of incubation. This prevents this method being used to establish an accurate PAK1 half-life. It does suggest, however, that PAK1 is a stable protein *in vivo* and is unlikely to have a high turnover rate. To determine a PAK1 half-life, pulse-chase labelling could have been utilised. This involves growing cells in the presence of Cys/Met $^{35}S$ to label the proteins, before

removal and a series of set growth times. The protein of interest is then immunoprecipitated and the levels of radioactive protein determined on an SDS-PAGE gel. Changes in radioactivity with time allow an accurate measure of a proteins half-life.

N-acetyl leucyl-leucyl norleucinal (ALLnL) is a proteasome (and Calpain) inhibitor frequently used to determine whether a protein is targeted to the proteasome. Inhibition of protein degradation using ALLnL or the *Streptomyces* metabolite Lactacystin which is an irreversible proteasome inhibitor did not affect PAK1 protein levels. While PAK has been shown to play a role in the degradation of proteins in yeast (Chiroli et al., 2003) and in regulation of calpain-dependent degradation of E3b1 in cell lines (Chi et al., 2004), no data at present links PAK1 to degradation in a proteasomal or calpain dependent manner. This suggests that PAK1 is not regulated by degradation via the proteasome.

The slow turnover rate of PAK1 suggests that it is unlikely that cessation of degradation would account for the marked increase in PAK1 protein levels after CSF-1/TNF$\alpha$ stimulation. An increase in protein translation is a possible mechanism by which PAK1 could be upregulated. One possibility is the presence of an Internal Ribosome Entry Site (IRES) within the 5'-UTR of the PAK1 mRNA. It was recently shown that the anti-apoptotic protein X-linked inhibitor of apoptosis protein (XIAP) contains such a sequence (Holcik, 2003) which allows the rapid increase in XIAP protein when the cellular protein synthesis is inhibited due to cellular stress. Other proteins that have been found to be translated from mRNAs containing an IRES sequence include transcription factors, growth factors and at least one kinase in protein kinase C $\delta$ (PKC$\delta$) (Morrish and Rumsby, 2002; also see review Vagner et al., 2001). Interestingly, the PAK binding protein $\beta$-PIX has had an isoform identified which is translated from an IRES within its mRNA (Rhee et al., 2004). However, the presence of IRES sequences in mRNAs has been questioned (Kozak, 2005).

Recent research has shown that p53 mRNA binds to the ribosomal protein L26 and nucleolin after DNA damage which resulted in an increased rate of p53 translation (Takagi et al., 2005). This mechanism offers the possibility that CSF-1 and TNF$\alpha$ may enhance PAK1 protein levels by inducing an increased rate of PAK1 translation rather than an induction of PAK1 mRNA transcription. This was investigated by studying the effect that inhibition of protein translation had upon CSF-1-induced PAK1 protein upregulation. Inhibition of translation using cycloheximide did not prevent the CSF-1-induced increase in PAK1 levels. This suggests that upon stimulation, new PAK1 protein is not being synthesised or cycloheximide does not inhibit the mechanism of translation that PAK1 undergoes. Data has shown that recovery of PAK1 protein levels after phorbol ester stimulation in HepG2 cells was inhibited by cycloheximide (Gujdar et al., 2003).

CSF-1 induced regulation of PAK1 protein levels does not seem to be controlled by changes in transcription or by conventional translational methods. One possible method for cells to alter protein levels is through post-translational modifications. Several post-translational modifications have been identified and multi-site modifications have been implicated in the coordination of intracellular signalling and protein function (see review (Yang, 2005)). The most common modification reported is phosphorylation and PAK has had a number of phosphorylation sites identified although no evidence has suggested they are responsible for protein stability (Bagheri-Yarmand et al., 2001; Zhou et al., 2003). The modification acetylation has been shown to promote p53 stability *in vivo* (Ito et al., 2001; Rodriguez et al., 2000) and may be critical for p53 function. There is no evidence of PAK1 acetylation in the literature and although preliminary studies suggested that PAK1 may be acetylated (data not shown), the results were inconclusive and incomplete. Numerous other modifications have been discovered *in vivo*, including sumoylation, ubiquitination and citrullination. Ubiquitination has been shown to be involved in protein activity and degradation (Wilkinson, 2000), whilst sumoylation (small ubiquitin-like modifier, SUMO) is believed to influence the cell cycle, genome integrity and act as a ubiquitin antagonist (see review (Hay, 2005)). Citrullination, however, does not involve the ligation of a

molecule onto the protein. A family of peptidylarginine deiminase enzymes (PAD) have been found to convert arginine residues within proteins to citrulline residues, a non-standard amino acid not incorporated into proteins during translation. Citrullination can therefore alter a proteins secondary and tertiary structure resulting in possible changes to the proteins intermolecular interactions, its ability to form new interactions and its susceptibility to proteolysis (see review (Vossenaar et al., 2003)). The ability of these post-translation modifications to alter a proteins stability, interactions and activity suggest a possible route by which PAK1 may be regulated. A population of modified PAK1 protein may also explain the presence of a doublet when using the anti-PAK1 antibody (Figure 3.1).

Another possible mechanism for the increase in PAK1 protein levels could be a change of protein localisation, with PAK1 going in to the cytosolic fraction at later time points of stimulated cells. Investigation of PAK1 levels in the pellet fraction of cell lysate from both starved and stimulated cells showed only low levels of PAK1 present with no changes observed upon stimulation (data not shown). This suggests that PAK1 is not being released from a different cellular compartment after stimulation.

It is possible that we observe enhanced PAK1 levels after stimulation because of a problem with the antibodies used. Potentially, the N-terminal site recognised by the anti-PAK1 antibody and the C-terminal site recognised by the C-19 anti-groupA PAK antibody could be masked in unstimulated cells. Upon stimulation with CSF-1, the masked sites may become available for antibody recognition and binding. It seems unlikely, however, that two separate antibodies that target different regions of the protein would both have their recognition sites masked in unstimulated cells. At present, it cannot be ruled out though.

Cellular stimulation with cytokines has been shown to induce PAK1 phosphorylation and activation (Adam et al., 1998). CSF-1 stimulation of BMMs showed that PAK phosphorylation increases between 2 and 5 minutes before reducing again around 10 minutes. This indicates that CSF-1 induces

the activation of PAK as well as the upregulation of PAK1 protein levels. However, the antibody used to investigate PAK activation is not specific. The primary sequence of PAK1, PAK2 and PAK3 around the critical threonine residue that indicates activation is identical suggesting that the antibody will recognise all the activated PAK isoforms. However, the antibody has been used in the literature as an indicator of PAK1 activation downstream of PDGF (Weber et al., 2004). The Mw of the bands seen on the western blot suggest that they are likely to be of a PAK1 origin; however, it is hard to be conclusive. To further investigate whether the activated isoform identified, a PAK1 kinase assay could be performed. This involves the immunoprecipitation of PAK1 with a PAK1 specific antibody and addition of PAK1 to a kinase buffer using myelin basic protein (MBP) as a substrate. Use of [$\gamma$-$^{32}$P] ATP allows the level of phosphate incorporation on to MBP to be established indicating the levels of active PAK1 present (Adam et al., 1998).

Activation of PAK1 has been shown to regulate the cytoskeleton. In HeLa cells, activated PAK1 was shown to induce loss of actin stress fibres and reorganise focal complexes (Manser et al., 1997). However, macrophages have a unique actin cytoskeleton and do not possess actin stress fibres. In macrophages, CSF-1 induces membrane protrusions and ruffling (Kheir et al., 2005) in a Rac1-dependent manner (Wells et al., 2004). To confirm the responses seen with CSF-1 stimulation of macrophages, BMMs were stimulated at time points where PAK1 activity was at its maximum and elevated PAK1 levels observed. Membrane ruffling was observed throughout the early time points correlating with PAK1 activity but was reduced by 1 hour after stimulation. At 2 hours after stimulation, however, the level of membrane ruffling was markedly increased again, concurrent with the raised levels of PAK1 protein. This possibly suggests that PAK1 plays a role in membrane ruffling and the increased PAK1 protein levels results in a fresh induction of ruffling. A number of possible mechanisms have been described that could allow PAK1 to influence membrane ruffling. These include PAK1 regulation of LIMK activity which stabilises actin via cofilin (Edwards et al., 1999), PAK1 regulation of actin-myosin contractility through phosphorylation

of MLCK (Sanders et al., 1999) or through Filamin A (FLNa) which is reported to be involved in PAK1 activation, aiding the activation of LIMK as well as being an actin-binding protein which cross-links actin filaments (Vadlamudi et al., 2002). These signalling pathways suggest possible mechanisms where an increase in PAK1 protein levels may influence membrane ruffling and cytoskeletal reorganisation in general.

Western blotting data, however, suggests that PAK1 is not activated at late time points after CSF-1 stimulation when PAK1 levels are elevated. This suggests that PAK1 is not affecting membrane ruffling via its downstream targets LIMK, MLCK or Filamin A at these time points. This raises the possibility that PAK1 is acting as a scaffold protein for other effectors which promote membrane ruffling. PAK1 has been implicated as a scaffold protein before to form a complex which activated Cdc42 via $\alpha$-PIX, which in turn lead to localised Cdc42 activation of PAK1 (Li et al., 2003). It is, therefore, possible that PAK1 forms a membrane complex at later CSF-1 stimulation time points which induces membrane ruffling. It is also possible that PAK1 activation in ruffling cells is localised and at a relatively low level so it is missed during western blotting.

Removal of PAK1 from the system would allow further investigation into its role in membrane ruffling. This could be achieved by use of RNAi (see reviews (Hammond, 2002; Hannon, 2005)), by generation of a knockout mouse or through the use of chemical inhibitors. Small molecule inhibitors of the PAK family are not currently available although research is being undertaken to design one. In the mean time, groups have been using a number of constructs to disrupt the signalling pathway. These include the use of point mutations to make dominant negative PAK1 (Frost et al., 2000), expressing the PAK1 auto-inhibitory domain (Mazumdar and Kumar, 2003) and development of PAK-specific peptide inhibitors derived from the ATP antagonist K252a (Nheu et al., 2002; Nheu et al., 2004). The most specific of these methods would be the generation of a $PAK1^{-/-}$ mouse from which BMMs could be cultured. Fortunately, Dr. J. Chernoff at the Fox Chase Cancer Centre in Philadelphia, USA recently generated a PAK1-null mouse.

The mouse was viable (Dr J. Chernoff, personal communication) and the macrophages were made available for research.

In summary, CSF-1 stimulation of starved BMMs leads to an early (2-5 minutes) activation and a later (2-3 hours) increase in PAK1 protein levels which appears to be independent of both changes to transcription and translation. PAK1 is a stable protein with a low turnover rate implicating that regulation of its degradation is unlikely to produce the increase in protein observed after stimulation. The increase in PAK1 protein appears to correlate with increased levels of membrane ruffling although PAK1 activity is not enhanced at this time. This suggests a possible scaffold role for PAK1 in later time point membrane ruffling.

# Chapter 4: Role of PAK1 in CSF-1-induced signalling.

## 4.1: Introduction

CSF-1 stimulation of BMMs has been shown to promote the activation of a number of signalling pathways including ERK, Akt and Rac (Grill and Schrader, 2002; Pixley and Stanley, 2004) and also the induction of cytoskeletal reorganisation, producing membrane ruffles on the cell surface (Wells et al., 2004). PAKs are effectors of Rac and Cdc42 signalling (Bagrodia et al., 1995b; Manser et al., 1994) and are activated by CSF-1 (Chapter 3). PAKs role in macrophages has not been analysed previously but a number of PAK-dependent signalling pathways have been identified in other cell types. One of the early pathways identified as a target of PAK1 was the p38 MAPK pathway (Zhang et al., 1995), a stress activated pathway involved in the inflammatory response. Since this discovery, PAK has also been implicated in regulation of ERK (Frost et al., 1996) and JNK (Bagrodia et al., 1995a). This regulation is believed to occur via a number of mechanisms including phosphorylation and activation of c-Raf (Arai et al., 2005; Beeser et al., 2005; Edin and Juliano, 2005), phosphorylation and activation of MEK1 (Slack-Davis et al., 2003), activation of the MAP kinase kinases MKK3 and MKK6 (Lee et al., 2001) and through regulating formation of a MEK1-ERK complex (Eblen et al., 2002). Other methods of regulation have also been reported indicating the complexity of this signalling pathway (Dard and Peter, 2006).

As well as playing a role in regulating MAPKs the PAK kinases have a number of targets that regulate cytoskeletal dynamics. PAK1 has been implicated in the regulation of the actin cytoskeleton through phosphorylation of LIM kinase (LIMK), which inactivates its target protein, the actin depolymerising factor cofilin, resulting in increased actin stability (Edwards et al., 1999). PAK1 also regulates actomyosin contractility through phosphorylation and inhibition of MLCK (Sanders et al., 1999) whilst PAK2 has been reported to phosphorylate MLC directly (Goeckeler et al., 2000). PAK1 has also been implicated in the regulation of microtubule stability

through phosphorylation and inactivation of the microtubule-destabilising protein Op18 (stathmin) (Daub et al., 2001; Wittmann et al., 2004).

Links between PAK and various signalling pathways have been elucidated using dominant negative (DN) and constitutively active (CA) PAK mutants. These are not ideal for the study of PAK function as they are capable of interfering with a number of signalling pathways by sequestering proteins from the system. Other groups have used the PAK auto-inhibitory domain to bind and prevent PAK activation; however, this will inactivate all group A PAKs (Beeser et al., 2005) and may potentially have off-target effects by binding more than just the PAK proteins. To study the role PAK1 plays in these signalling pathways, cells from a $PAK1^{-/-}$ mouse were utilised. Dr. Jonathon Chernoff's laboratory at the Fox Chase Cancer Centre, Philadelphia, engineered the mouse on a Sv129 genetic background that was back-crossed onto a C57B6 genetic background. Using bone marrow from this mouse to culture primary macrophages, it was possible to investigate the role PAK1 has in a number of signalling pathways.

## Results

### 4.2: $PAK1^{-/-}$ bone marrow cells differentiate into macrophages and have a normal morphology

CSF-1 stimulates the activity of PAK1 in BMMs (Figure 3.6) whilst data in the literature implicates PAK1 in MAPK signalling and hence, gene transcription (Vadlamudi et al., 2000). This raises the possibility that PAK1 may affect cellular differentiation, as CSF-1 stimulation is essential for myeloid stem cell differentiation into a macrophage. Evidence for PAK1's role in differentiation is highlighted in research suggesting that PAK1 is involved in the differentiation of mouse mammary glands *in vivo* (Wang et al., 2003).

Western blotting of BMM lysates using the C19 group A PAK (PAK1, 2 and 3) antibody or an anti-PAK1 antibody confirmed that PAK1 protein was not

detectable in PAK1$^{-/-}$ cells (Figure 4.1A). The C19 antibody also detected no PAK3 protein in WT or PAK1$^{-/-}$ BMMs.

To ascertain whether PAK1$^{-/-}$ bone marrow cells could differentiate into macrophages in the presence of CSF-1, cell surface levels of the macrophage marker, F4/80, were investigated on WT and PAK1$^{-/-}$ cells. F4/80 is a member of the EGF transmembrane 7 family of receptors and is specifically expressed on myeloid cells but particularly macrophages. Until recently, the receptor had little known about its function but new research suggests it may have a role in the development of antigen-specific regulatory T cells that suppress antigen-specific immunity (see review (van den Berg and Kraal, 2005)). A FITC-conjugated F4/80 antibody was used to stain WT and PAK1$^{-/-}$ cells after 7 days of CSF-1 stimulated differentiation (Figure 4.1B). WT and PAK1$^{-/-}$ cells expressed similar levels of F4/80 on their cell surface indicating PAK1 is not required for BMM differentiation.

PAK1 is implicated in signalling to both the actin cytoskeleton and microtubules, but no obvious defects were detected in the morphology of the PAK1$^{-/-}$ BMMs in growth medium (Figure 4.1C) suggesting that PAK1s role in signalling to the cytoskeleton is either minor or only evident in cells undergoing a change in morphology such as during migration.

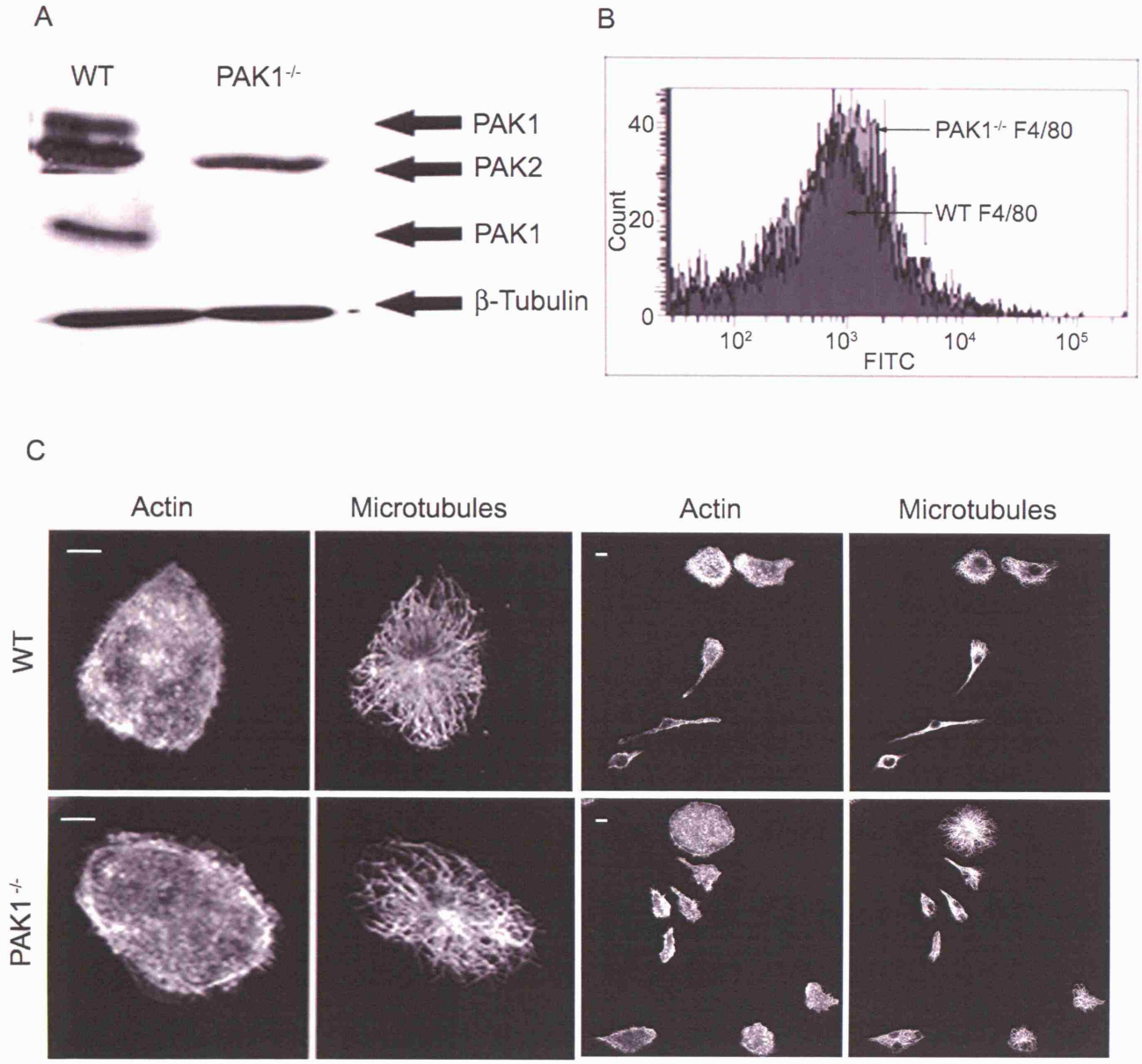



**Figure 4.1:** PAK1$^{-/-}$ cells differentiate into macrophages

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium. A) Western blot analysis of PAK1 and PAK2 levels in WT and PAK1$^{-/-}$ BMM using a group A C19 PAK antibody (upper panel) and a PAK1-specific antibody (middle panel). β-tubulin levels were used as a loading control. B) BMMs were suspended in FACS buffer and stained with either a FITC-conjugated F4/80 antibody or a FITC-conjugated IgG2b negative control. Cell surface F4/80 expression in WT and PAK1$^{-/-}$ cells was analysed by flow cytometry. C) WT and PAK1$^{-/-}$ BMMs were grown on glass coverslips for 24 hours before fixation and staining with TRITC-phalloidin (actin) and FITC-β-tubulin (microtubules). Confocal images of the actin cytoskeleton and microtubules are shown. Results shown are representative of 3 separate western blot experiments and two separate flow cytometry experiments.

## 4.3: PAK1 is required for maximal activation of the ERK, JNK and p38 MAPK pathways upon CSF-1 stimulation of macrophages

PAK1 has been implicated in the activation of the MAPK pathways downstream of a number of receptors in various cell types, mostly using DN and CA mutants (Bagrodia et al., 1995a; Frost et al., 1996). To determine whether PAK1 regulates activation of MAPKs in macrophages, CSF-1 was used to stimulate WT and PAK1$^{-/-}$ BMMs.

MAPK pathway activation was investigated by western blotting using phosphorylation specific antibodies. ERK1/2 activation was determined using a phospho-Thr202/Tyr204 antibody, p38 activation with a phospho-Thr180/Tyr182 antibody and JNK activation with a phospho-Thr183/Tyr185 antibody. These antibodies specifically recognise the phosphorylated active forms of the kinases allowing determination of the activation levels.

In growth medium WT BMMs had low levels of phosphorylated-ERK (P-ERK), P-p38 and P-JNK (Figure 4.2). CSF-1 starvation led to a reduction in P-ERK and P-JNK but little change in P-p38. Upon CSF-1 stimulation, active levels of ERK, p38 and JNK all increased peaking at 5 minutes for ERK and JNK and remained elevated until 10 minutes for p38.

The time course of stimulation was similar in WT and PAK1$^{-/-}$ BMMs. However, the phosphorylated kinase levels were lower throughout the period of stimulation in PAK1$^{-/-}$ BMMs (Figure 4.2) suggesting that PAK1 is required for the maximal activation of each MAPK pathway but is not essential for the initiation of MAPK signalling.

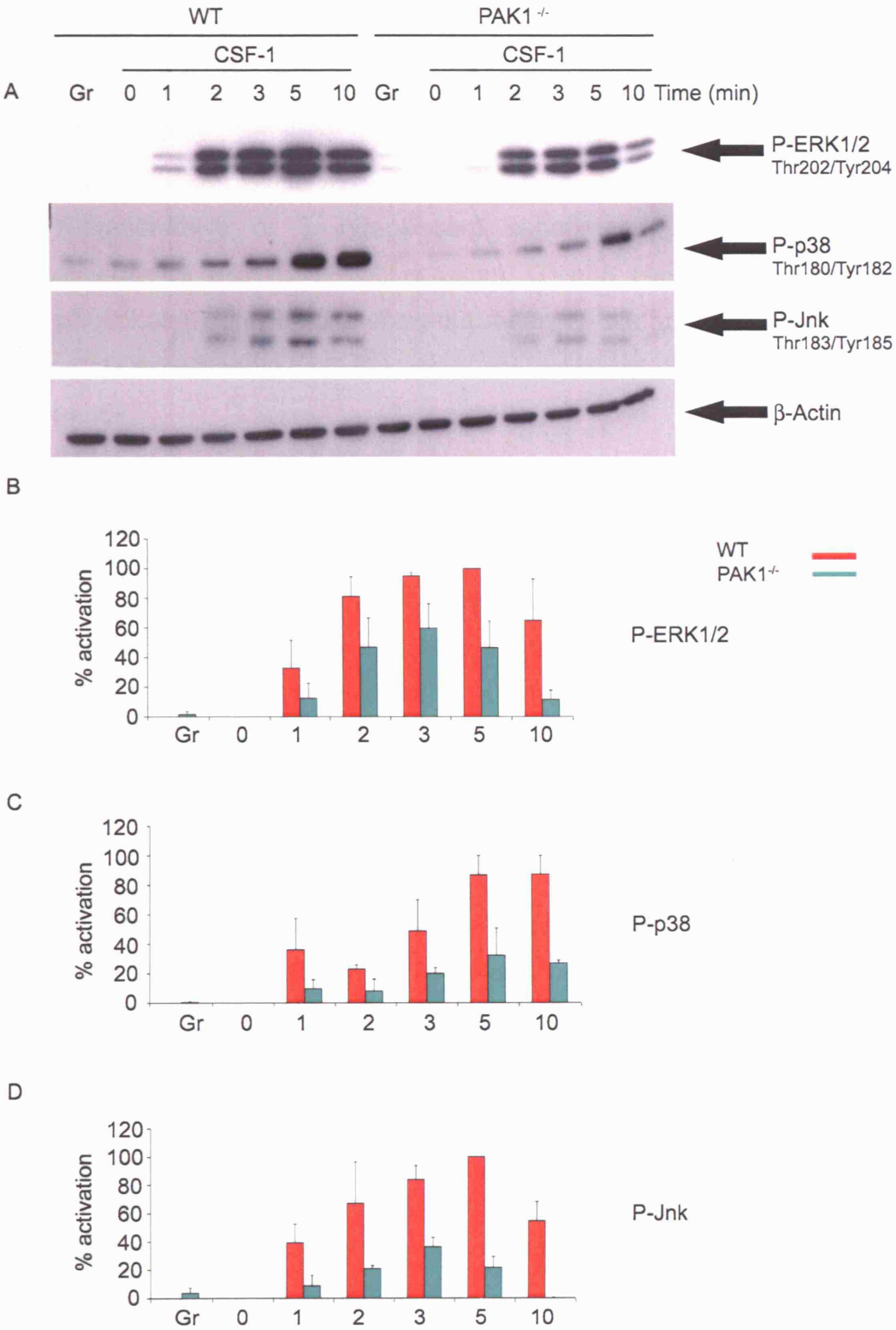



**Figure 4.2:** PAK1$^{-/-}$ BMMs have reduced MAPK activation upon CSF-1 stimulation.

WT and PAK1$^{-/-}$ were either maintained in growth medium (Gr) or starved of

CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for between 0 and 10 min then cells were lysed. Proteins were separated by SDS-PAGE, then western blotted with A) phosphorylation-specific antibodies for ERK1/2, p38 and JNK proteins to identify active MAPK levels after stimulation. $\beta$-Actin levels were used as a loading control. All western blots are representative of 3 independent experiments. B-D) Densitometer quantification of ERK1/2 phosphorylation, C) p38 phosphorylation and D) JNK phosphorylation. Results show the mean +/- s.e.m, data is pooled from 3 separate experiments.

## 4.4: PAK1 regulation of ERK1/2 activation is not via phosphorylation of c-Raf or MEK1

A number of mechanisms for PAK1 regulation of the ERK pathway have been described. These include the phosphorylation of c-Raf by PAK at Ser338 and the phosphorylation of MEK1 at Ser298. Recent evidence has shown that PAK1 phosphorylates c-Raf at Ser338 and MEK1 at Ser298 in response to growth factor stimulation of NIH-3T3 and HeLa cells (Beeser et al., 2005).

To determine whether changes to c-Raf or MEK1 phosphorylation were responsible for the reduction in ERK activation in PAK1$^{-/-}$ BMM, antibodies specific for the c-Raf and MEK1 sites phosphorylated by PAK1 were used on western blots. CSF-1 stimulation of WT and PAK1$^{-/-}$ BMMs led to an increase in both c-Raf Ser338 and MEK1 Ser298 phosphorylation, and no differences were observed between WT and PAK1$^{-/-}$ BMMs (Figure 4.3). PAK1 is therefore not required for the phosphorylation of c-Raf and MEK1 at sites previously shown to be PAK1 targets downstream of growth factor signalling. PAK1 regulation of CSF-1-induced ERK activation is, therefore, independent of c-Raf and MEK1 stimulation although it is possible that other previously unidentified phosphorylation sites within c-Raf and MEK1 are being phosphorylated by PAK1.

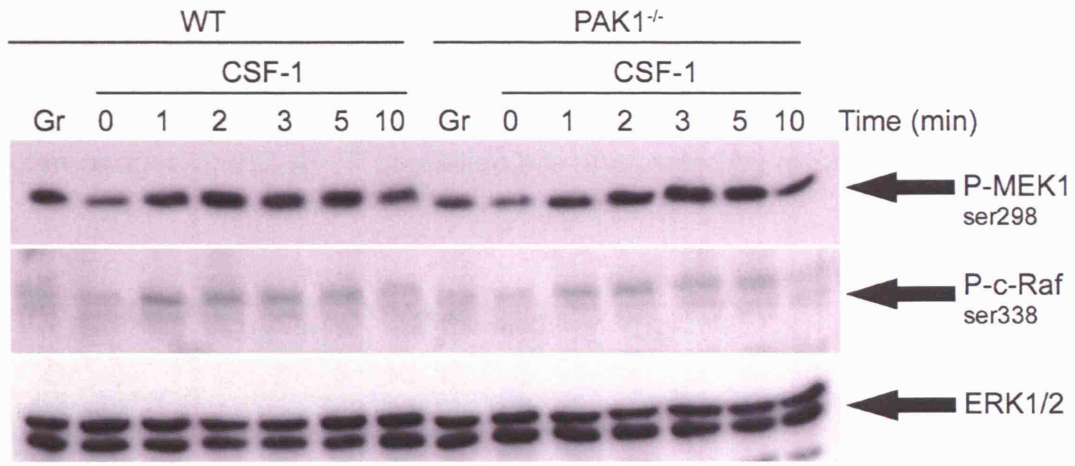



**Figure 4.3:** PAK1$^{-/-}$ reduction in MAPK signalling after CSF-1 reduction is not due to loss of MEK1 or c-Raf signalling.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for between 0 and 10 min and cells were lysed. Proteins were separated by SDS-PAGE and western blotted with antibodies to phosphorylated c-Raf and MEK1. Total ERK1/2 was used as a loading control. Western blots are representative of two separate experiments.

### 4.5: Akt activation downstream of CSF-1 is unaffected by loss of PAK1

PI3-K and PAK1 are likely to regulate a number of common targets downstream of CSF-1 signalling, including MAPKs. A major target of activated PI3-K is Akt (PKB) (Gingery et al., 2003). Activation of Akt by PI3-K has been shown to play a role in cell survival through inhibition of apoptosis (see review (Stambolic et al., 1999)) but also in the regulation of the ERK pathway. PI3-K-Akt led to the inhibition of B-Raf and a reduction in MEK-ERK signalling (Guan et al., 2000). However, PI3-K has also been implicated in activation of ERK (Bilancio et al., 2006).

To determine whether CSF-1 signalling was generally reduced in PAK1$^{-/-}$ BMMs; an anti-phospho-Akt Ser473 antibody was used to indicate activated levels of Akt after CSF-1 stimulation. CSF-1 stimulation led to an increase in

phospho-Akt Ser473 within 1 minute and remained activated at a high level between 2 and 10 minutes (Figure 4.4). However, no changes were evident between WT and PAK1$^{-/-}$ BMMs suggesting that PI3-K-Akt signalling in macrophages and CSF-1R signalling are unaffected by removal of PAK1.

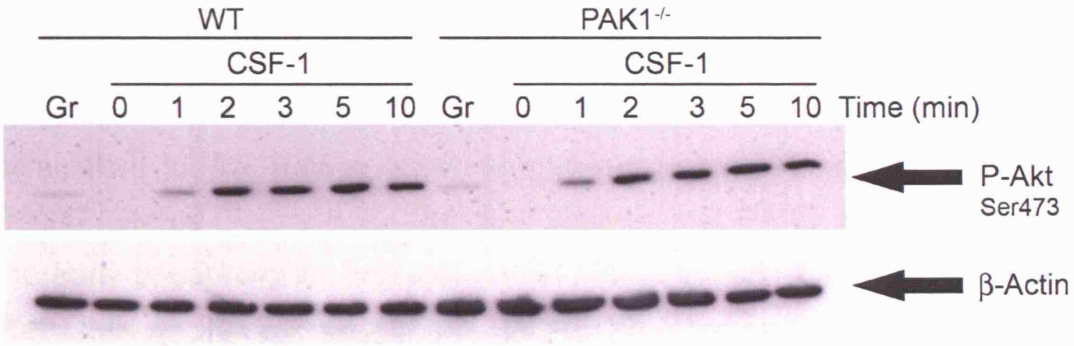



**Figure 4.4:** PAK1 does not regulate Akt activation.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for 0 to 10 min and cells were lysed. Cell lysates were separated by SDS-PAGE and western blotted using a phosphorylation-specific antibody targeting Akt Ser473 to monitor Akt activity after stimulation. *β*-actin was used as a loading control and the western blot shown is representative of three separate experiments.

### 4.6: PAK1 is required for CSF-1-induced LIMK phosphorylation

PAK1, as a target of Rac and Cdc42, is believed to play an important role in reorganisation of the cytoskeleton (see reviews (Bagrodia and Cerione, 1999; Bokoch, 2003)). A key target for PAK-mediated regulation of the actin cytoskeleton is believed to be LIM kinase 1 (LIMK1). PAK1 phosphorylates Thr508 a critical residue in the activation loop of LIMK1 resulting in its activation. Active LIMK1 phosphorylates its target, cofilin, an actin depolymerising factor, (Edwards et al., 1999) resulting in increased actin filament stability.

To evaluate whether PAK1 regulates LIMK1 after CSF-1 stimulation in macrophages, an antibody specific for phosphorylated Thr508 in LIMK1 and Thr505 in LIMK2 was used as a probe for LIMK activation.

LIMK activation was not detectable in growing and CSF-1 starved BMMs, with or without PAK1. However, upon CSF-1 stimulation, LIMK activation was visible after 2 minutes in WT BMMs but absent in PAK1$^{-/-}$ BMMs. A second, lower increase in activity was detected 5 and 10 minutes after stimulation in WT BMMs, which was also present but slightly reduced in PAK1$^{-/-}$ BMMs (Figure 4.5). The data suggest that PAK1 is responsible for the early phosphorylation of LIMK after CSF-1 stimulation. It also indicates that LIMK is phosphorylated in a biphasic manner and that this may be a result of separate signalling pathways, since the later activation of LIMK is only slightly reduced in PAK1$^{-/-}$ BMMs. This could potentially be due to the absence of the earlier, PAK1 dependent, activation. It is not possible to distinguish between LIMK1 and LIMK2 as both isoforms have similar molecular weights although data in the literature suggests PAK1 regulates LIMK1 activity (Edwards et al., 1999).

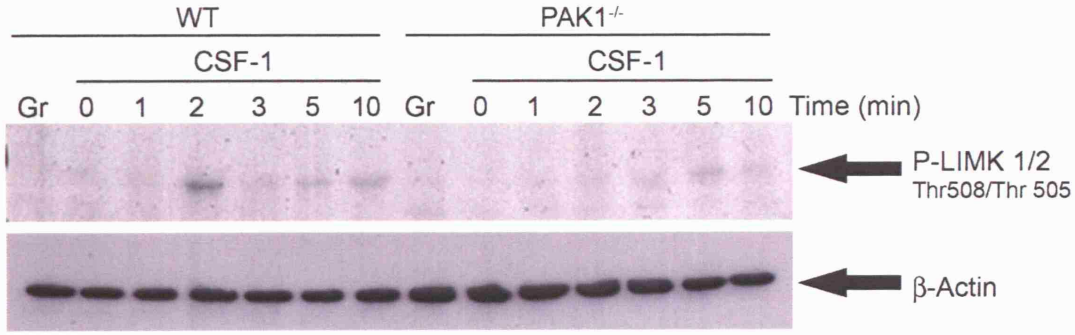



**Figure 4.5:** PAK1 regulates phosphorylation of LIMK.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for 0 to 10 min before cell lysate was made. Cell lysates were separated by SDS-PAGE and western blotted with phosphorylated LIMK-specific antibodies. *β*-actin was used as a loading control. Western blots are representative of two separate experiments.

### 4.7: Loss of PAK1 results in an upregulation of MLC phosphorylation

PAK1 can regulate the actin cytoskeleton via its activation of LIMK1. Another potential mechanism is via its ability to inhibit myosin light chain kinase (MLCK) which phosphorylates MLC and thereby regulates actin-myosin contractility. PAK1 regulation of MLC phosphorylation has already been described (Chapter 1.3.3.1 and Figure 1.11). It was reported that PAK1 is capable of regulating the phosphorylation of MLC through inhibition of both MLCK (Sanders et al., 1999) and MLCP (Takizawa et al., 2002).

PAK1 regulation of MLC phosphorylation was determined by western blotting with a phospho-MLC Ser19 antibody. In WT BMMs, little or no P-MLC was detected and it did not increase upon CSF-1 stimulation (Figure 4.6) suggesting that CSF-1 does not control MLC phosphorylation at these early time points. However, in PAK1$^{-/-}$ BMMs, the P-MLC levels were elevated in growing cells with lower levels present in starved and CSF-1 stimulated cells. No response to CSF-1 was observed.

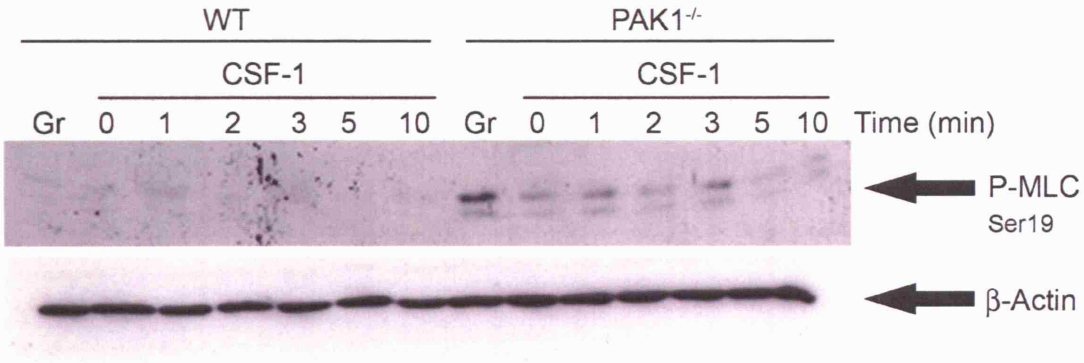



**Figure 4.6:** PAK1, but not CSF-1, regulates myosin light chain phosphorylation.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for 0 to 10 min and western blotting analysis of phosphorylated MLC levels was performed. $\beta$-actin levels were used as a loading control. Western blot shown is representative of three separate experiments.

### 4.8: PAK1 regulates CSF-1-induced Op18 phosphorylation

PAK1 can regulate the microtubule network via the microtubule destabilising protein Op18 (Daub et al., 2001; Wittmann et al., 2004). PAK1 phosphorylates Op18 on Ser16 inhibiting its activity (Wittmann et al., 2004). A number of other regulatory kinases target Op18, including Calcium-Calmodulin Dependent Protein Kinase (CaM II), Cyclin Dependent Kinase 1 and 2 (Cdk1, Cdk2) and cAMP Dependent Protein Kinase (PKA) (see review (Cassimeris, 2002)).

Western blotting with a phospho-Ser16 Op18 antibody was used to investigate whether CSF-1 or PAK1 affected Op18 phosphorylation. CSF-1 stimulation of WT BMMs induced an increase in phospho-Op18 between 3 and 10 minutes (Figure 4.7). In PAK1$^{-/-}$ BMMs, however, phosphorylation of Op18 was reduced although some phosphorylation was still observed. This suggests PAK1 is a major, but not the only, kinase targeting Op18 downstream of CSF-1.

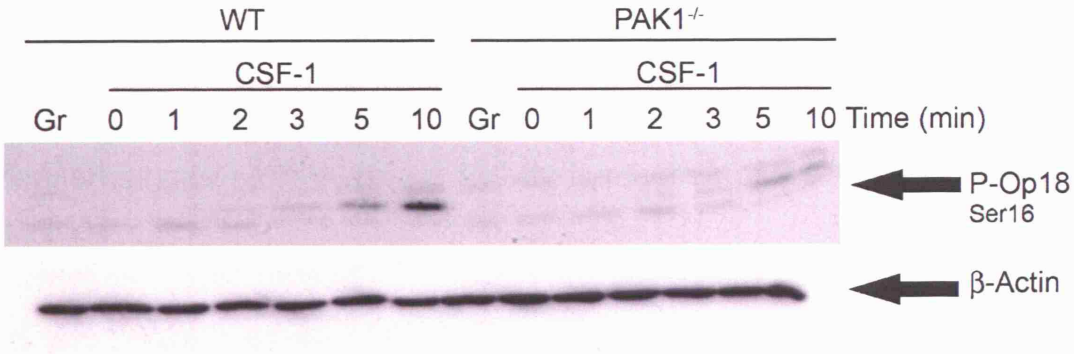



**Figure 4.7:** PAK1 regulates Op18 phosphorylation.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for 0 to 10 min and lysed. Cell lysates were resolved by SDS-PAGE and western blot analysis of phosphorylated Op18 protein levels was performed. $\beta$-actin levels were used as a loading control. The western blot is representative of three separate experiments.

## 4.9: CSF-1-induced phosphorylation of paxillin is enhanced in PAK1$^{-/-}$ BMMs

Paxillin is a multidomain scaffolding protein that is a crucial component of focal adhesions at the leading edge of migrating cells. Phosphorylation of paxillin at tyrosine residues 31 and 118 has been implicated in the formation of focal adhesions in migrating cells (Nakamura et al., 2000) and was shown to be vital for MM1 cancer cell migration (Iwasaki et al., 2002). Phosphorylation of these residues was also observed to suppress RhoA activity through binding of p120RasGAP, thereby decreasing its binding and inhibition of p190RhoGAP (Tsubouchi et al., 2002). The residues Tyr31 and Tyr118 are also the binding sites for the adaptor protein CrkII. When phosphorylated, this allows binding of the RacGEF DOCK180 and activation of Rac1 (Valles et al., 2004).

PAK and paxillin are linked via the adaptor proteins GRK Interactor 1 (GIT1) and PAK Interacting Exchange Factor (PIX). GIT1 binding to paxillin in focal adhesions targets PIX and PAK1 to the complex and can result in adhesion disassembly (Zhao et al., 2000). GIT1 targeting of PAK1 to adhesions was also shown to induce activation of PAK1 in a Rac/Cdc42-independent manner suggesting PAK kinase activity is required at the adhesion (Loo et al., 2004).

An anti phospho-paxillin Tyr118 antibody was used to determine whether CSF-1 and PAK1 are involved in the regulation of paxillin. Western blotting showed that CSF-1 stimulation induced phosphorylation of paxillin at Tyr118, with a marked increase 1 min after stimulation. With time, a number of higher bands become visible representing various serine/threonine phosphorylation states of paxillin. In PAK1$^{-/-}$ BMMs, there is a small increase in the phosphorylation species after 10 minutes stimulation. This suggests that PAK1 may play a role in inhibiting the phosphorylation of paxillin after 10 minutes of CSF-1 stimulation as loss of PAK1 results in greater paxillin phosphorylation.

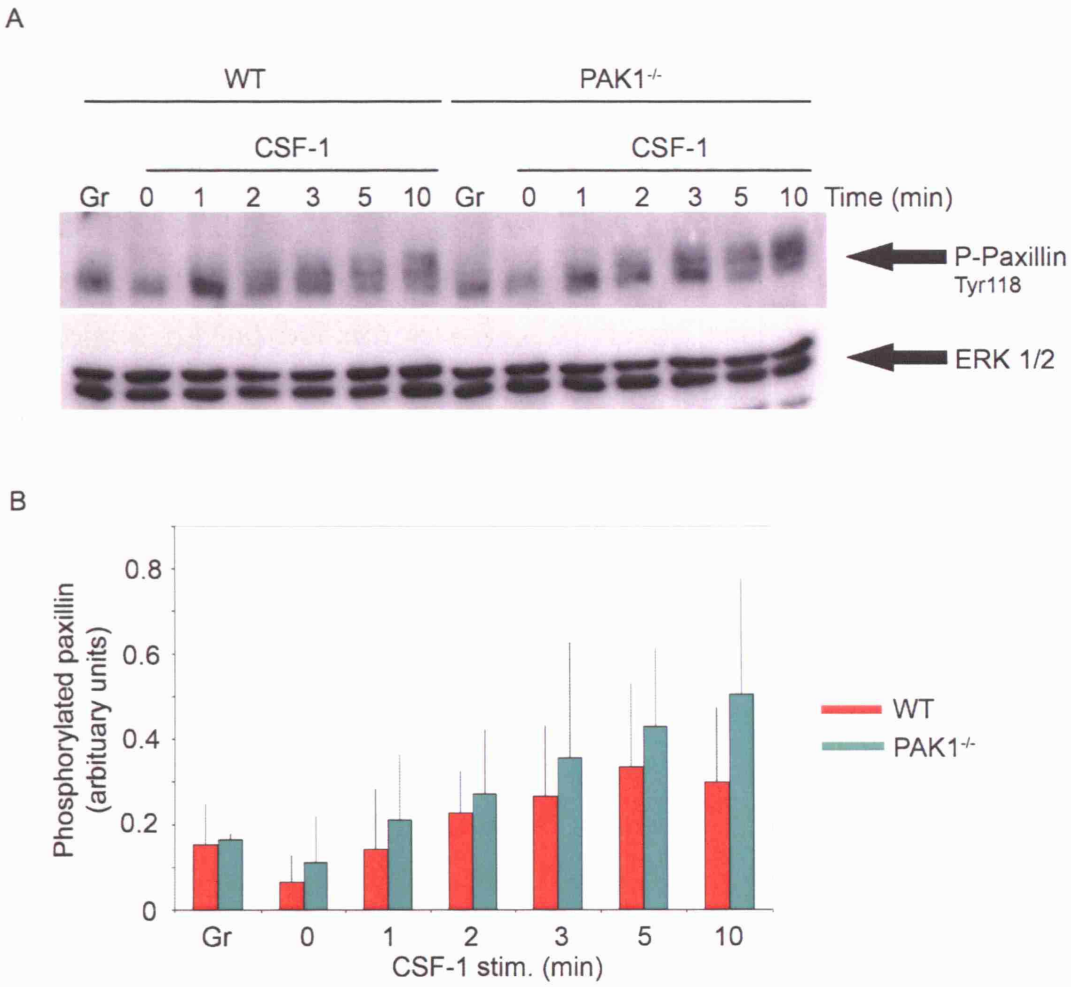


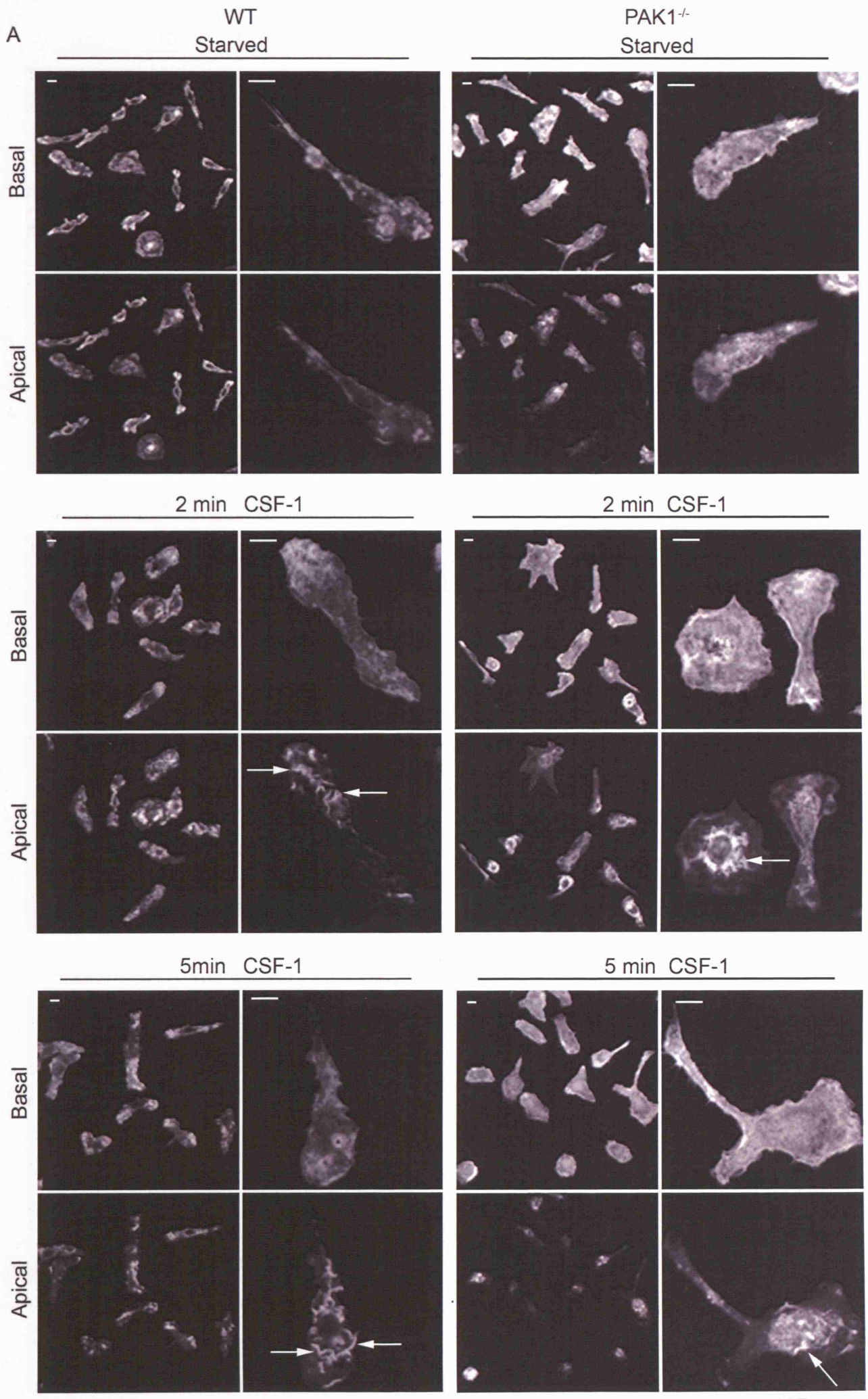

**Figure 4.8:** Loss of PAK1 leads to a slight increase in paxillin phosphorylation.

WT and PAK1$^{-/-}$ BMMs were maintained in growth medium (Gr) or starved of CSF-1 for 16 hours. Starved BMMs were stimulated with 33 ng/ml CSF-1 for 0 to 10 min and were then lysed. A) Lysate was separated by SDS-PAGE and western blot analysis was performed to investigate levels of phospho-paxillin Tyr118. Total ERK1/2 levels were used as a loading control. The western blot is representative of two separate experiments. B) Quantification of normalised phospho-paxillin levels by densitometry analysis. Results show mean +/- s.d from two separate experiments.

## 4.10: PAK1$^{-/-}$ BMMs have reduced membrane ruffling in response to CSF-1 stimulation

CSF-1 stimulation of CSF-1 starved BMMs induces apical membrane ruffling (Figure 3.8) and this is dependent on Rac1 and Rac2 (Wells et al., 2004; Wheeler et al., 2006). In Rac1$^{-/-}$ BMMs, deletion of Rac1 led to a reduction of membrane ruffling but not in migration speed suggesting ruffling is not essential for migration. As Rac1 is a direct effector of PAK1 and PAK1 regulates the actin cytoskeleton through LIMK and MLCK, a role for PAK1 in membrane ruffling was investigated.

PAK1$^{-/-}$ BMMs were grown on glass coverslips and then starved of CSF-1. Upon stimulation of CSF-1, some membrane ruffling is observed, however it is to a reduced degree when compared to WT BMMs. WT BMM ruffling was frequently seen to encompass large areas of the apical surface and were common on the leading lamellipodium and edge of cells with a migratory phenotype. However PAK1$^{-/-}$ BMMs had reduced levels of ruffling which tended to be localised to the central area of the cell, typically above and around the nucleus. Much less ruffling was observed on the apical surface of lamellipodia.


A
WT
Starved
PAK1-/-
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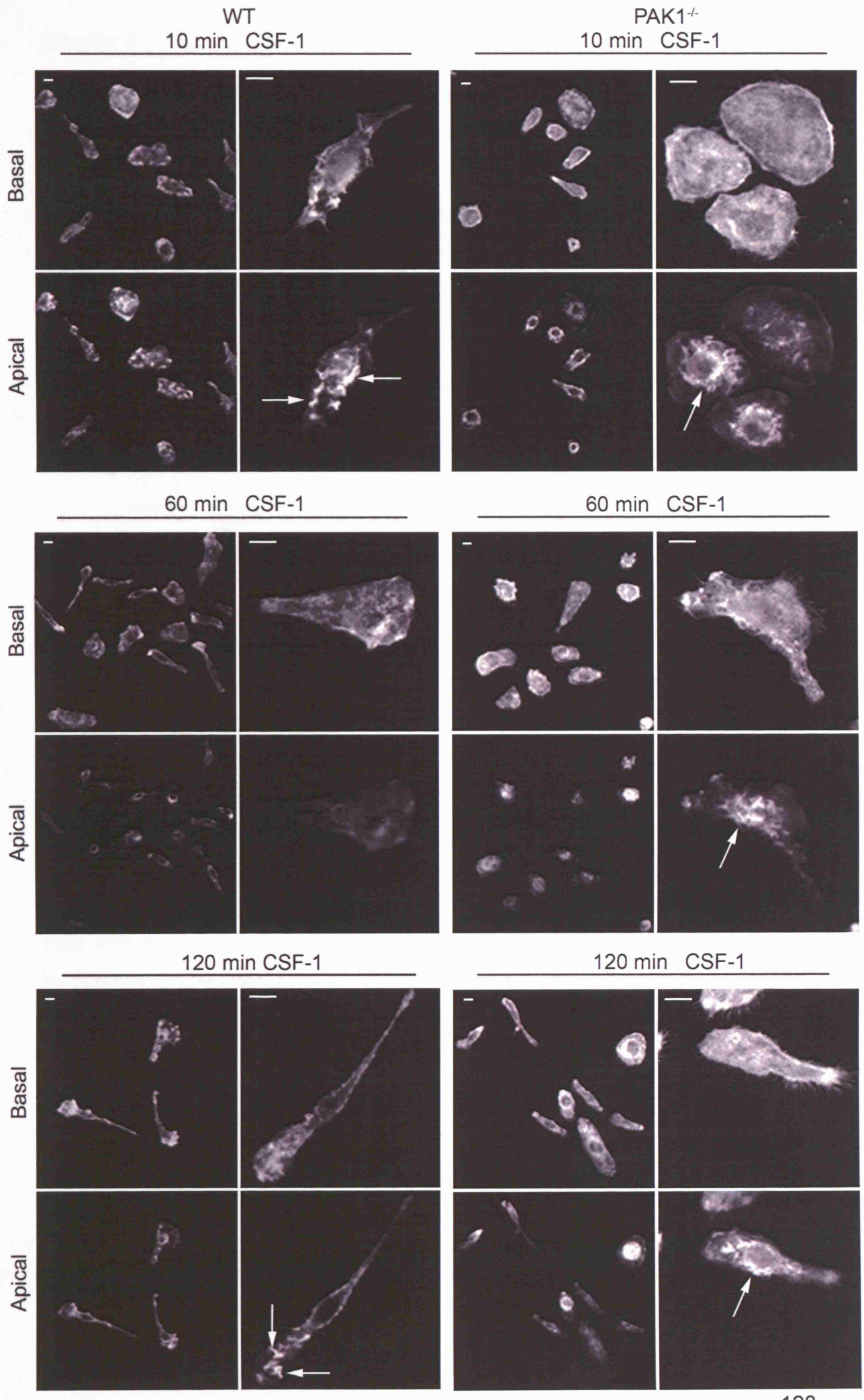
WT
10 min CSF-1
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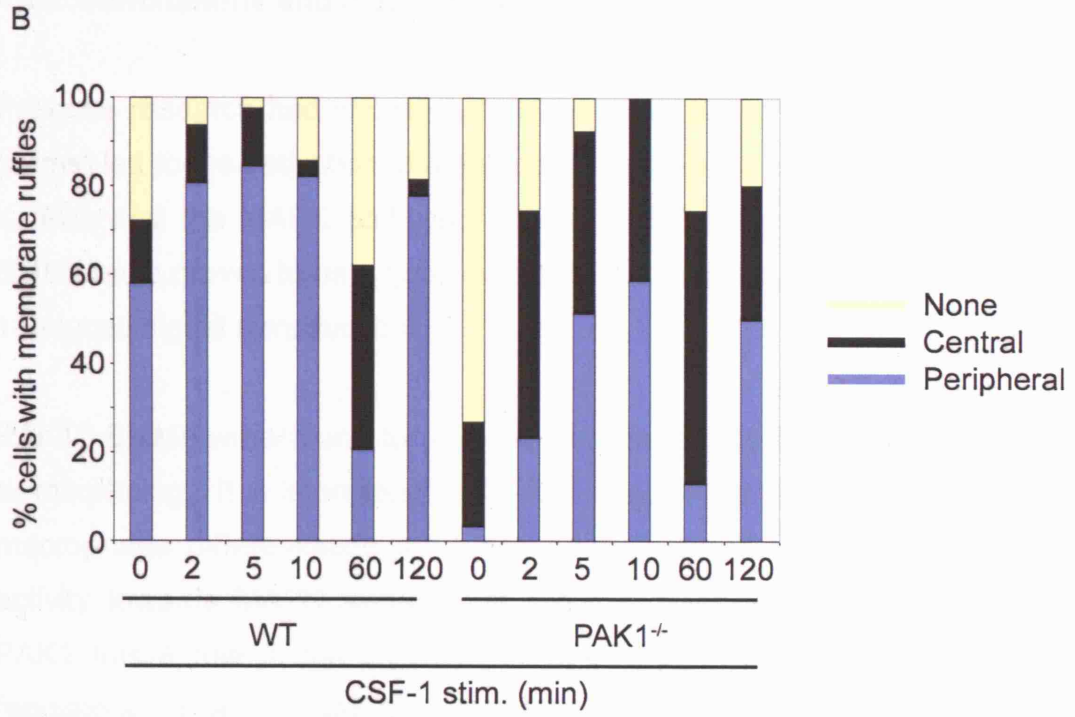



**Figure 4.9:** PAK1$^{-/-}$ BMMs produce fewer peripheral membrane ruffles

WT and PAK1$^{-/-}$ BMMs were cultured on glass coverslips in growth medium before starvation of CSF-1 for 16 hours. BMMs were then stimulated with 33 ng/ml CSF-1 for between 0 and 120 min. A) Cells were stained with TRITC-phalloidin to show actin filaments and confocal images of the basal and apical views of the cells were acquired. B) Quantification of apical membrane ruffling in WT and PAK1$^{-/-}$ BMMs through scoring for the presence or absence of ruffles in the cell periphery or centre. Results show percentage of cells with each phenotype. n = 213 (WT), 215 (PAK1$^{-/-}$), pooled from two independent experiments.

## 4.11: Conclusions and discussion

Previous research had shown that CSF-1 activation of its receptor CSF-1R (c-fms) led to the activation of a number of signalling pathways including PI3-K, Rac and the MAPK pathways (Pixley and Stanley, 2004). The $PAK1^{-/-}$ BMMs have proven to be a good model for studying the role of PAK1 in CSF-1-induced signal transduction.

$PAK1^{-/-}$ BMMs were found to have no defects in differentiation, proliferation or morphology. It is interesting that PAK1 is not essential for CSF-1-induced macrophage differentiation as it has been reported that persistent MEK1 activity towards MAPK signalling is required (Gobert Gosse et al., 2005). PAK1 has a role in the regulation of the MAPK pathways which are key regulators of a number of transcription factors potentially involved in differentiation (Bagrodia et al., 1995a; Frost et al., 1996; Zhang et al., 1995) and is implicated in mouse mammary gland differentiation (Wang et al., 2003). It was also interesting to observe that growing $PAK1^{-/-}$ BMMs have no clear morphological defects compared to WT BMMs. $Rac1^{-/-}$, $Rac2^{-/-}$ and $Rac1/Rac2^{-/-}$ BMMs all exhibited morphological changes (Wells et al., 2004; Wheeler et al., 2006b). This suggests that PAK1 is not essential for Rac1 and Rac2 mediated regulation of BMM morphology.

PAK1 was found to be required for optimal activation of all three MAPK pathways as implicated in the literature (Chapter 1.3.6). It is surprising to see that PAK1 appears to have such a wide affect upon the MAPK pathways in macrophages. Previous reports have used a diverse number of systems and cell types to identify PAK1 as a regulator of each of the MAPK pathways. However, no reports have shown that PAK1 regulates all three MAPK downstream of a single stimulus so it appears that CSF-1 induction of the MAPKs in macrophages is critical and that PAK1 plays a role in this regulation. It is still unclear, however, how PAK1 regulates the JNK and p38 pathways.

The MAPK pathways have been implicated in the regulation of a number of cellular processes including cell proliferation (see reviews (Fang and Richardson, 2005; Nishina et al., 2004)). The fact that the $PAK1^{-/-}$ mouse is viable (Ten Klooster et al., 2006) and the ability to culture $PAK1^{-/-}$ BMMs suggest that PAK1 does not affect cell proliferation significantly. However, PAK1 has been implicated in the control of proliferation in breast cancer cells via a hyperactive Rac3 pathway (Mira et al., 2000). PAK1 was also shown to be involved in upregulation of cyclin D1 levels in an NF-$\kappa$B dependent manner (Balasenthil et al., 2004) and its activity was targeted by the tumour-suppressor protein merlin (Xiao et al., 2005). These data suggest that although $PAK1^{-/-}$ BMMs can still proliferate at a rate comparable to WT BMMs in culture, PAK1 may play a role in proliferation through its ability to upregulate cyclin D1 levels via the transcription factor NF-$\kappa$B. Potentially, PAK1 may help regulate proliferation only after specific stimuli.

CSF-1 stimulation of the phosphorylation of MEK1 or c-Raf showed no change in WT and $PAK1^{-/-}$ BMMs despite being reported PAK1 targets (Arai et al., 2005; Beeser et al., 2005; Edin and Juliano, 2005; Slack-Davis et al., 2003).

Growth factor stimulation of MAPKs involves PAK1 phosphorylation of c-Raf (Beeser et al., 2005) and MEK1 (Slack-Davis et al., 2003) although PAK1 phosphorylation of MEK1 promotes activation of MEK1 by c-Raf rather than directly activating it (Coles and Shaw, 2002). However, PAK1s role in c-Raf stimulation is controversial (R. Marais, personal communication), an opinion supported by the observation that despite loss of PAK1 resulting in a reduction of MAPK signalling, no change in c-Raf or MEK1 phosphorylation was observed. It is possible that PAK redundancy means PAK2 can phosphorylate c-Raf and MEK1 upon CSF-1 stimulation although it is also possible that another kinase is responsible for c-Raf and MEK1 phosphorylation in BMMs. However, that does not explain the observed reduction in ERK signalling. PAK1 has been shown to associate directly with ERK2 upon adhesion where it may act as a scaffold protein for Raf, MEK and ERK (Sundberg-Smith et al., 2005). Although, formation of this complex

was after cell adhesion to fibronectin, it is possible that it is also formed after CSF-1 stimulation. This could explain the reduction in ERK activation as loss of PAK1 would result in the complex not being formed and so a resulting loss of Raf-MEK-ERK signalling. PAK1 has also been shown to promote the formation of a MEK1-ERK2 complex upon adhesion to fibronectin (Eblen et al., 2002). Use of an anti-MEK1 and an anti-ERK1/2 antibody to try and co-immunoprecipitate the complex was unsuccessful however (data not shown). PAK1 has also been implicated downstream of Ras signalling in transformation of Rat-1 fibroblasts (Tang et al., 1997). Ras activates the MAPK pathway through a Raf-MEK pathway and activates the tyrosine kinase ETK which was shown to interact and activate PAK1 (Bagheri-Yarmand et al., 2001; He et al., 2004). This is another potential pathway through which PAK1 may regulate the MAPK pathway.

The CSF-1 receptor, upon activation, induces the activity of class IA PI3-Ks (Vanhaesebroeck et al., 1999) which target the kinase Akt promoting PAK1 (Menard and Mattingly, 2004) and B-Raf phosphorylation (Guan et al., 2000). No difference in phosphorylated Akt levels were observed between WT and $PAK1^{-/-}$ BMMs showing that changes in MAPK signalling is not a result of generally reduced CSF-1R signalling. Akt phosphorylation of B-Raf reduces its activation of ERK suggesting that the loss of MAPK signalling observed could be a result of enhanced Akt activity. The active Akt levels observed suggest that loss of ERK signalling is not due to a defect in Akt signalling.

PAK1 is frequently implicated in the regulation of the cytoskeleton. A key PAK1 target is LIMK1, whose phosphorylation is believed to promote its activation and promote phosphorylation of the actin depolymerising factor (ADF), cofilin (Edwards et al., 1999). Early CSF-1-induced stimulation of LIMK activation was prevented in $PAK1^{-/-}$ BMMs whilst a later activation seen at 5 to 10 min was less affected by PAK1 suggesting that it may only be the initial activation of LIMK1 that is PAK1-dependent. The kinases ROCK or MRCK$\alpha$ have also been implicated in LIMK1 regulation (Chapter 1.3.3.1) so the later activation of LIMK1 may be dependent upon these.

Downregulation of LIMK1 is performed by a family of protein phosphatases called Slingshot (Soosairajah et al., 2005), the same phosphatases that dephosphorylate and activate cofilin (Niwa et al., 2002) (Chapter 1.3.3.1). It has been reported that PAK4 can phosphorylate and inhibit slingshot (Soosairajah et al., 2005) and phosphorylate LIMK1 (Dan et al., 2001). BMMs do not express PAK4 (Figure 3.1) but it is possible that PAK1 is also capable of inhibiting Slingshot activity via phosphorylation; although this was not tested by Soosairajah *et al.* PAK1 regulation of LIMK1 activity may therefore be a dual process with phosphorylation of LIMK1 and inhibition of Slingshot. Comparisons can be drawn between this proposed mechanism and ROCK regulation of MLC. ROCK can stimulate MLC contractility through direct phosphorylation but can also inhibit MLCP through its subunit MYPT1. This is an interesting possibility but needs further investigation.

Cofilin and the other members of the ADF/Cofilin family of proteins regulate actin filament dynamics by binding ADP-F-actin and producing a twist in the filament. This changes the stability of the filament causing it to depolymerise, allowing an increase in the actin filament 'treadmilling' which is the driving force behind cell migration (see review (Paavilainen et al., 2004)). It was reported that active cofilin at the leading edge is vital for maintaining cellular polarity (Dawe et al., 2003) although LIMK1 activity and its inhibition of cofilin were shown to be critical for early induction of lamellipodia formation in SDF-1$\alpha$ stimulated Jurkat cells (Nishita et al., 2005). The role of cofilin and LIMK1 in actin dynamics and polarisation suggest that PAK1 may play a role in processes such as cell migration and spreading (Chapter 5).

PAK1 has been shown to regulate actomyosin contractility through MLC phosphorylation levels (Sells et al., 1999) (Figure 1.11). PAK1 may also be capable of regulating phosphorylated MLC levels indirectly via the MAPK pathway. It was shown that adhesion and growth factor stimulation of ERK1/2 via MEK lead to phosphorylation of MLCK (Klemke et al., 1997). However, unlike PAK1 induced MLCK phosphorylation, ERK1/2 phosphorylation of MLCK increased its activity and promoted MLC phosphorylation.

MLC phosphorylation is not induced upon BMMs activation with CSF-1 but PAK1$^{-/-}$ BMMs showed elevated phospho-MLC levels. This suggested that PAK1 inhibits the phosphorylation of MLC in BMMs under growing conditions. PAK1 probably regulates actomyosin contractility via direct phosphorylation of MLCK in BMMs and not via signalling to MLCP or ERK. The data also showed that actomyosin contractility is not a pathway influenced by CSF-1 stimulation of BMMs, although it may be at later time points.

Regulation of the microtubule network by PAK1 is proposed to be through phosphorylation of the microtubule destabilising protein Op18 at Ser16 (Daub et al., 2001; Wittmann et al., 2004). Consistent with this, phosphorylated Op18 levels were reduced in PAK1$^{-/-}$ BMMs although it might not be the only kinase responsible. The low levels of phosphorylation observed after 5 to 10 minutes in PAK1$^{-/-}$ BMMs suggests other kinases target Op18 in addition to PAK1. One possibility is that PAK2 can partially compensate for the loss of PAK1 and phosphorylate Op18.

PAK1 appears to be a major regulator of Op18 downstream of CSF-1 as levels of Op18 Ser16 are significantly reduced in PAK1$^{-/-}$ BMMs. The fact that total loss is not observed suggests another kinase is also regulating Op18 phosphorylation. There are a number of kinases capable of regulating the activity of Op18 (Figure 4.11) and they may be responsible for the phosphorylation levels observed in PAK1$^{-/-}$ BMMs. Both PKA and CaMK IV/Gr have been identified as kinases that phosphorylate the Ser16 residue (Gradin et al., 1998; Melander Gradin et al., 1997) but have not been reported downstream of CSF-1 signalling. Alternatively, the MAPK proteins, p38 (Mizumura et al., 2006; Parker et al., 1998) and ERK (Lovric et al., 1998) may have a role to play, although no evidence has been shown of their ability to phosphorylate Ser16. This is an intriguing possibility, however, given PAK1 regulation of the MAPK proteins. Loss of PAK1 could reduce phosphorylation of Op18 both directly and indirectly via ERK and p38. However, at present this is speculation and further research is required to fully understand the mechanisms of Op18 regulation.

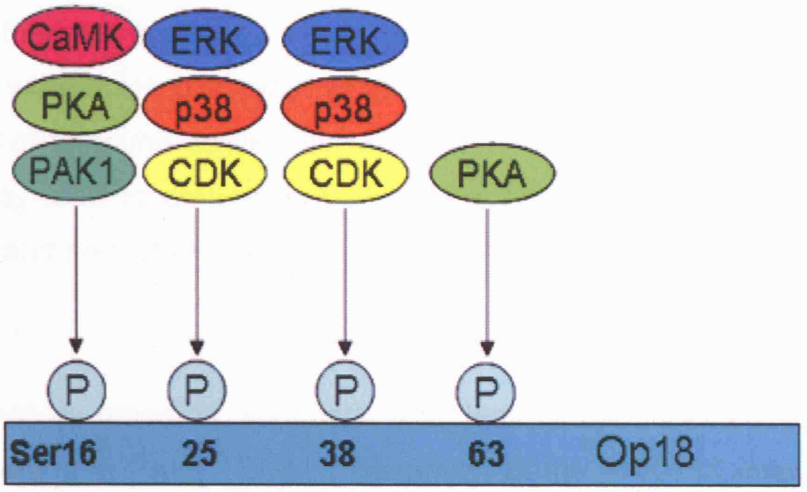



**Figure 4.11:** Regulation of Op18 activity through phosphorylation

Diagram showing the kinases reported to phosphorylate and regulate Op18 activity. PAK1, PKA and CaMK IV/Gr can all phosphorylate Op18 Ser16 which is believed to regulate Op18 activity. CDKs can phosphorylate Ser25 and Ser38 which is required for the G2/M transition (Larsson et al., 1995) as can ERK and p38 although a clear function for this has not been reported. PKA was reported to also phosphorylate Ser63 during its inactivation of Op18 activity.

Regulation of proteins that affect the actin and tubulin cytoskeleton by PAK1 suggests it plays a role in cell migration. However, changes in cell adhesion are also critical for co-ordinated migration. RhoA has been implicated in the formation of focal adhesions (Rottner et al., 1999), whilst Rac1 has been shown to induce both assembly of focal complexes (Allen et al., 1997) and disassembly of focal adhesions either through PAK signalling (Zhao et al., 2000) or through antagonism of RhoA signalling (Sander et al., 1999).

PAK regulation of adhesions (focal complexes) involves a number of adaptor proteins to appropriately target the protein and affect paxillin localisation. Phosphorylation of paxillin may therefore play a critical role in regulation of cell adhesions. CSF-1 induced phosphorylation of paxillin in WT and $PAK1^{-/-}$ BMMs but Tyr118 phosphorylation was slightly elevated in $PAK1^{-/-}$ BMMs after 10 minutes stimulation.

It is possible that the enhanced levels of phosphorylated paxillin seen in PAK1$^{-/-}$ BMMs were a result of reduced adhesion turnover or possibly due to a greater level of unbound active Rac which could recruit other targets. This could potentially lead to greater formation of adhesions via the Crk-GIT2-$\beta$PIX pathway and so higher levels of phosphorylated paxillin (Lamorte et al., 2003).

However, previous research suggests that less phosphorylated paxillin should be observed in PAK1$^{-/-}$ BMMs. Disruption of the PAK-PIX interaction in breast cancer cells led to a reduction of PIX and paxillin in focal adhesions (Stofega et al., 2004) although only total paxillin and not phosphorylated paxillin was observed in the study. It is possible that the differences observed are a consequence of changes in the breast cancer cells used by Stofega *et al.* and that ordinarily, loss of PAK1 results in a small reduction in the turnover of paxillin-containing focal complexes. Another possibility is that macrophages only produce small focal complexes and not the larger focal adhesions observed in a number of cell lines. Differences in their structure and regulation may explain the inconsistent data but further study of PAK1 regulation of focal complexes is required.

The formation of membrane ruffles upon growth factor stimulation is believed to be the induction of macropinocytosis, a process that contributes to both cellular growth and motility (Amyere et al., 2002). CSF-1 has been shown to stimulate BMM membrane ruffles in a Rac and Cdc42 dependent manner (Wells et al., 2004). However, disruption of both Rac1 and Cdc42 did not completely ablate ruffle formation suggesting other pathways are also involved downstream of CSF-1 signalling. One possibility that has been identified is RhoA induction of ruffling through its effector mDia (Kurokawa and Matsuda, 2005). The involvement of Rac1 and Cdc42 in this process, however, suggests a possible role for PAK1. Results showed that PAK1$^{-/-}$ BMM have reduced, but not absent levels of membrane ruffling. However, the ruffles observed in PAK1$^{-/-}$ BMMs were typically localised over the nucleus and not within the peripheral region as in WT BMM. Therefore, it is possible PAK1 is required for peripheral membrane ruffling but not for

centrally localised ruffling. PAK1 has previously been implicated in membrane ruffling through its target Filamin A (FLNa) (Vadlamudi et al., 2002) and also in macropinocytosis (Dharmawardhane et al., 2000). Microinjection of CA PAK1 induced membrane ruffling as well as filopodia in Swiss3T3 cells (Sells et al., 1997) suggesting PAK1 may regulate the formation of CSF-1 induced membrane ruffles in BMMs, possibly via MLCK and LIMK.

The downstream effectors of PAK1 in this pathway remain unclear. As well as FLNa (Dharmawardhane et al., 2000), MLCK has been shown to help regulate macropinocytosis downstream of the Fc*γ* receptor (Fc*γ*R) in macrophages (Araki et al., 2003) and LIMK1 was implicated in macropinocytosis. DN LIMK1 inhibits *Listeria* invasion protein, InlB, induced membrane ruffling (Bierne et al., 2001). This data suggests that PAK1 could regulate membrane ruffling downstream of Rac or Cdc42 by a number of differing pathways.

In summary, PAK1 is not essential for CSF-1-induced differentiation of mouse bone marrow cells into macrophages. However, PAK1 is required for maximal activation of ERK, p38 and JNK downstream of CSF-1 signalling, although the loss of ERK signalling does not appear to be due to reduced phosphorylation of c-Raf, MEK1.

As well as regulating the MAPK pathways, PAK1 may regulate the actin cytoskeleton through LIMK and MLCK and the microtubule network through phosphorylation of Op18. These changes could contribute to the reduction in membrane ruffling and increased paxillin phosphorylation that was also observed.

# Chapter 5: The role of PAK1 in motility and CSF-1-induced chemotaxis.

## 5.1: Introduction

Through regulation of cytoskeletal dynamics and adhesion turnover, the Rho GTPases co-ordinate cell migration (see reviews (Raftopoulou and Hall, 2004; Ridley, 2001; Ridley et al., 2003)). The individual roles of the different Rho GTPase family members are gradually being elucidated (Chapter 1.2.2.4).

PAK1 has been implicated as a mediator of cell migration downstream of Akt signalling (Zhou et al., 2003) and directional sensing towards C5a (Li et al., 2003) and CXCL1 (Wang et al., 2002). It is also believed to help regulate tracheal smooth muscle cell chemotaxis via p38 signalling (Dechert et al., 2001). A role for PAK1 in endothelial cell migration was also suggested, however, in this case, PAK1 is not required for lamellipodial extension but for the formation and turnover of adhesions at the front and rear of the cell (Kiosses et al., 1999).

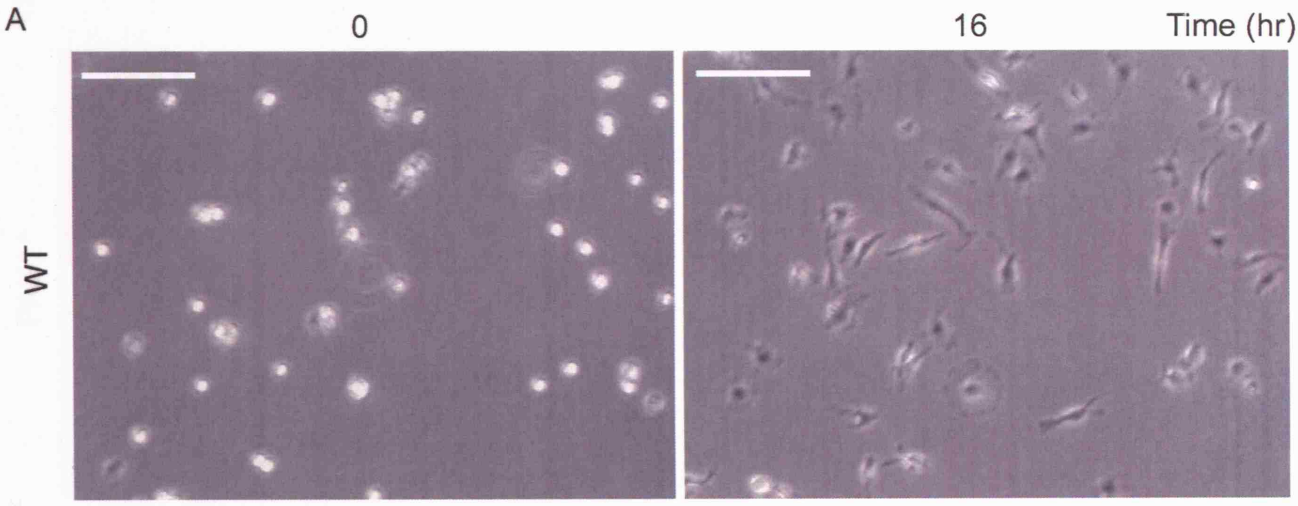
These reports, therefore, suggest a role for PAK in cell migration, however, most of the results were based on overexpression studies in various cell lines. As has been stated (Chapter 4.1), CA and DN mutants are not ideal for determining protein function due to their potential to sequester upstream and downstream binding partners and disrupt active protein localisation. The use of $PAK1^{-/-}$ BMMs should confirm with more certainty whether PAK1 is essential for macrophage migration. Bac1.2F5 macrophages (Allen et al., 1998) and BMMs (Vedham et al., 2005) chemotax towards CSF-1 allowing WT and $PAK1^{-/-}$ BMMs to be used to study the role PAK1 plays in CSF-1-induced migration.

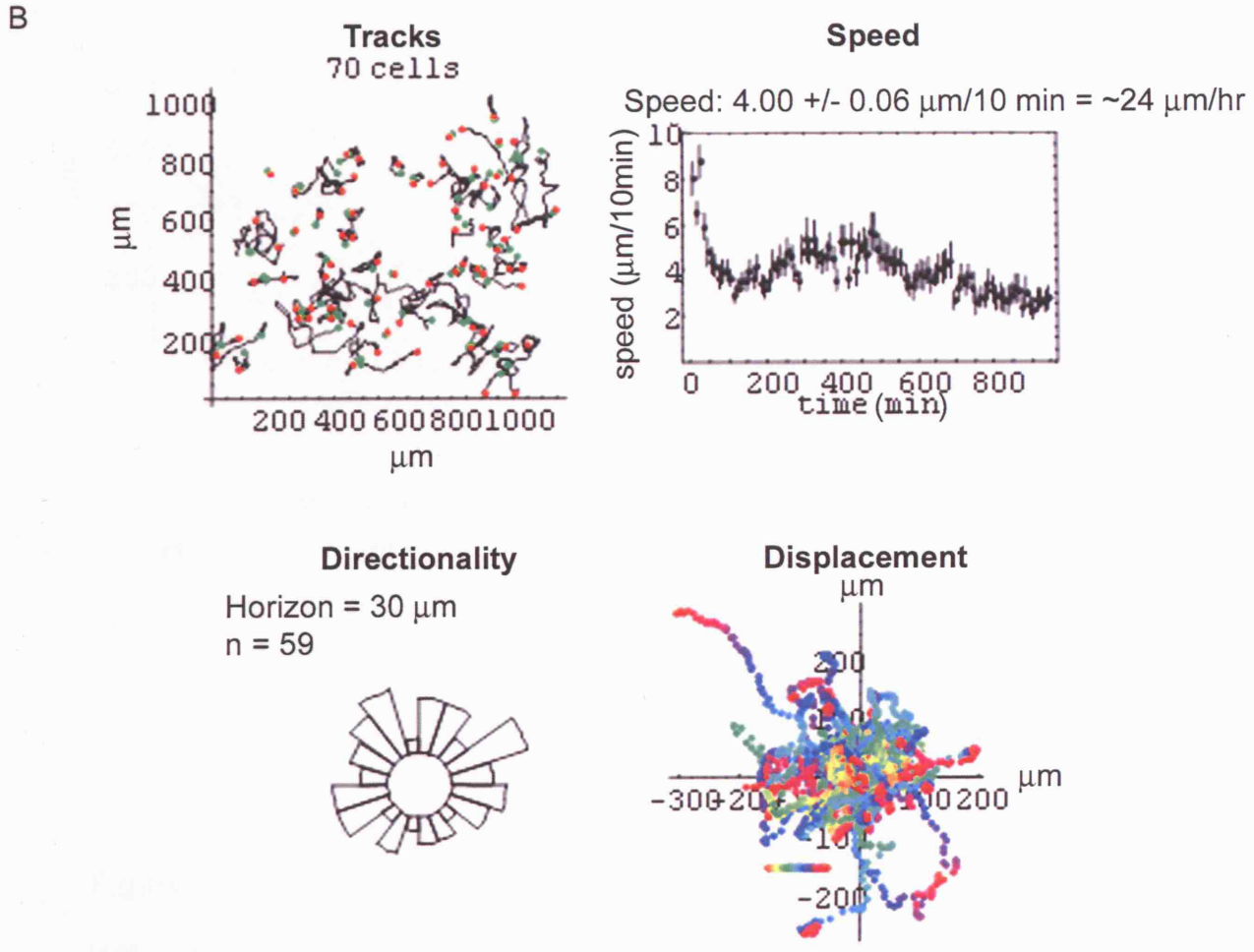
## Results

### 5.2: WT and PAK1$^{-/-}$ BMMs adhere, polarise and migrate

To investigate potential defects in PAK1$^{-/-}$ BMM migration, cell polarisation and migration in the absence of a chemical gradient (random migration) was studied first.

Upon plating WT BMMs in growth medium onto tissue culture plastic, the cells adhered, spread, polarised and migrated with a clear leading edge lamellipodium and retraction tail (Figure 5.1A and Movie 5.1). PAK1$^{-/-}$ BMMs responded similarly to WT BMMs (Figure 5.1C and Movie 5.2). WT and PAK1$^{-/-}$ BMMs showed frequent turning events consistent with the lack of a chemical gradient. The average speed of BMM migration was determined by tracking the cells in Motion Analysis and then further analysed in Mathematica 5.0. Analysis showed that WT and PAK1$^{-/-}$ BMMs both migrated with an average speed of ~ 24 $\mu$m/hour and with no significant directionality (Figure 5.1B and 5.1D), as expected in the absence of a gradient.


A
0
16
Time (hr)
WT


B
Tracks
70 cells
1000
800
600
400
200
µm
200 400 600 800 1000
µm
Speed
Speed: 4.00 +/- 0.06 µm/10 min = ~24 µm/hr
speed (µm/10min)
10
8
6
4
2
0 200 400 600 800
time (min)
Directionality
Horizon = 30 µm
n = 59
Displacement
µm
200
-300 -200
200
µm
-200

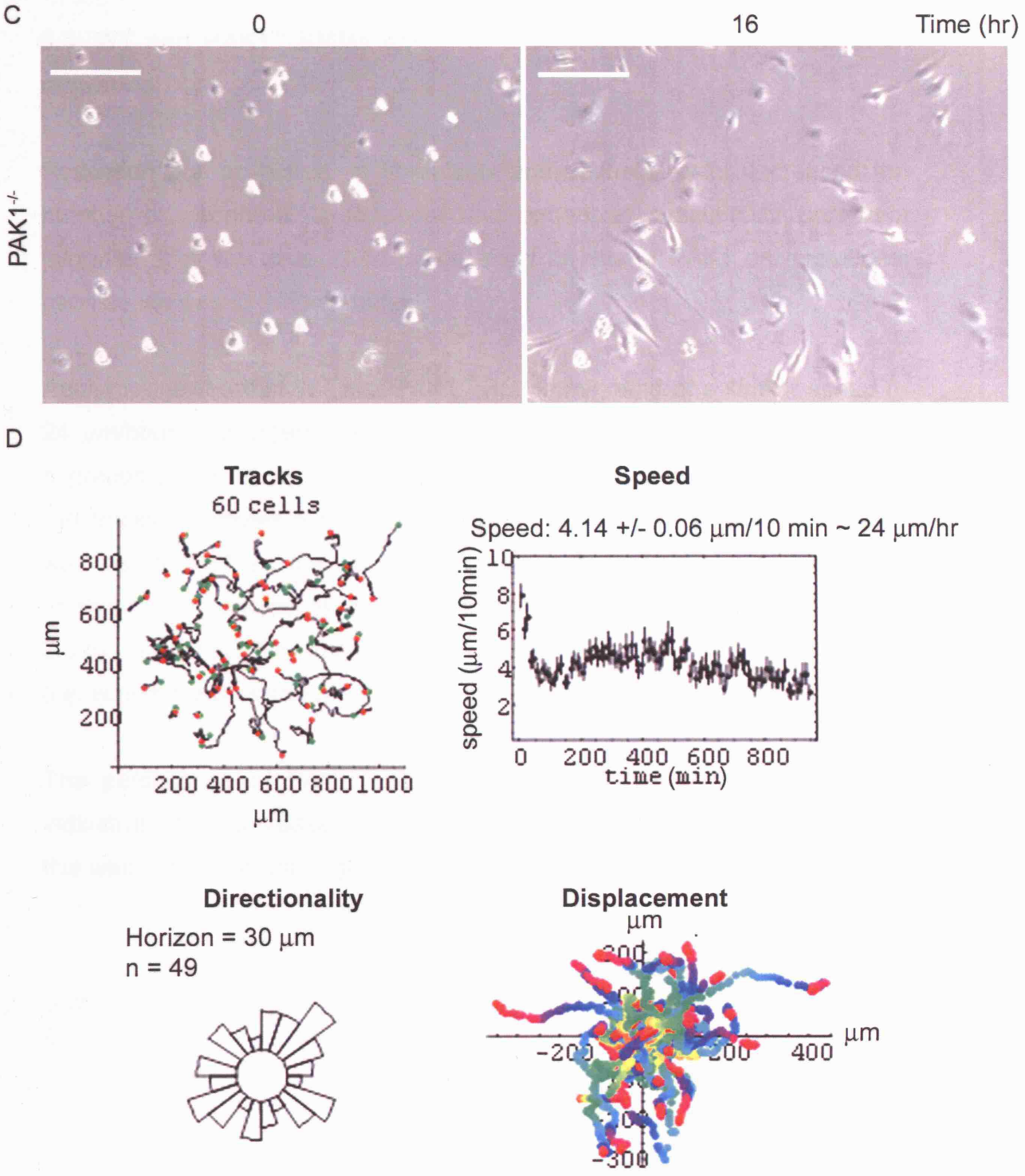



**Figure 5.1:** PAK1$^{-/-}$ BMMs migrate at the same rate as WT BMMs on plastic.

WT and PAK1$^{-/-}$ BMMs suspended in growth medium were plated onto tissue culture plastic. A, C) Timelapse microscopy was used to observe WT and PAK1$^{-/-}$ BMMs adhering and randomly migrating (Movies 5.1 and 5.2), bars = 100 µm. B, D) Cells in the movies were tracked using Motion Analysis software and analysed using Mathematica software. Images and tracking data are representative of two separate experiments. Horizon = distance cell must migrate to be included in analysis. The migration paths (displacement) are represented as a temperature scale, with the start of the path in green and the end in red.

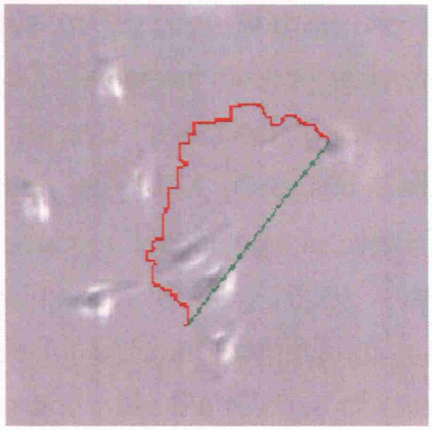
## 5.3: WT and PAK1$^{-/-}$ BMMs show no differences in the persistence of migration

Reduction of Rac activity in fibroblasts and epithelial cells decreased the number of peripheral lamellipodia and enhanced directionally persistent migration (Pankov et al., 2005). The effect of loss of PAK1 on migrational persistence was therefore studied.

Analysis showed that WT and PAK1$^{-/-}$ BMMs migrated at a similar speed (~ 24 $\mu$m/hour). To determine whether loss of PAK1 resulted in a change in migration persistence, the persistence of the cells was measured from the cell tracks of movies 5.1 and 5.2 (Figure 5.1B and 5.1D). The persistence was established as the cells displacement from its starting point to its finishing point divided by the total distance migrated by the cell (Chapter 2.5.6). The closer the quotient was to 1, the fewer times the cell had turned (i.e. was more persistent).

The persistence of PAK1$^{-/-}$ BMMs was slightly higher than WT BMMs, indicative of an increase in migratory persistence (Figure 5.2B), however, this was not statistically significant.

A



$$\text{Persistence} = \frac{\text{Displacement from starting point}}{\text{Total distance moved}}$$

B

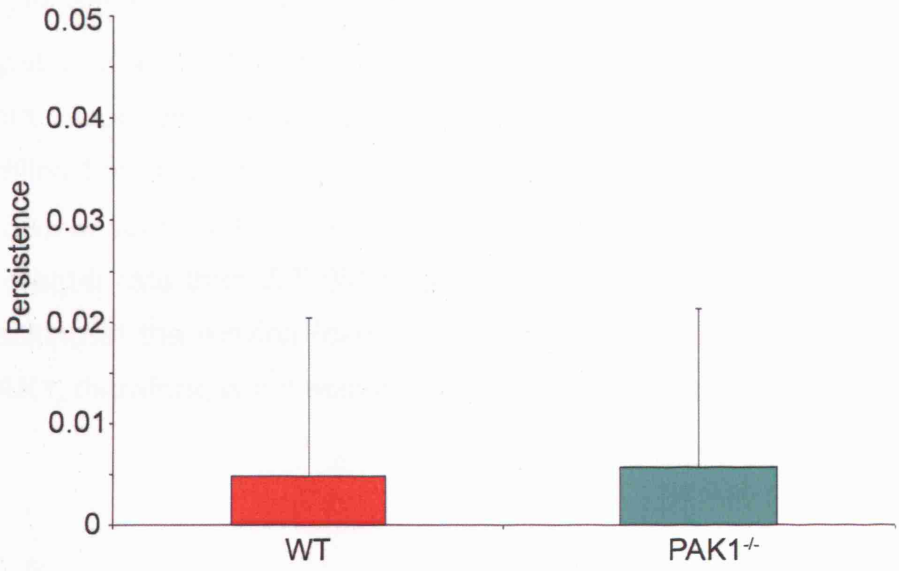



**Figure 5.2:** WT and PAK1$^{-/-}$ BMMs have similar persistence during migration.

Timelapse microscopy movies of WT and PAK1$^{-/-}$ BMMs in growth medium, spreading and migrating on plastic were acquired. A) Phase-contrast image of a migrating BMM. The migration path is highlighted in red and the displacement between its start and finishing point in green. B) Persistence for WT and PAK1$^{-/-}$ BMMs. Results show mean persistence +/- s.d. Data were pooled from two separate experiments (n=49) and showed no significant difference between the genotypes.

## 5.4: WT and PAK1$^{-/-}$ BMMs respond similarly in scratch assays

Timelapse microscopy showed no obvious defects in random migration (Figure 5.1). Another method to analyse cell migration is a scratch assay. Scratch assays have been used to implicate PAK in fibroblast protrusion polarisation via β-PIX (Cau and Hall, 2005) and have been adapted to a high-throughput technology to identify potential inhibitors of cell migration (Soderholm and Heald, 2005). Scratch assays are therefore a useful technique for studying cell migration in response to loss of cell-cell contact inhibition and possible release of chemokines and cytokines from damaged cells.

Confluent monolayers of WT and PAK1$^{-/-}$ BMMs were scratched with a pipette tip and cells filmed by timelapse microscopy. WT and PAK1$^{-/-}$ BMMs both responded to scratches in the monolayer by increasing membrane ruffling before extending lamellipodia and migrating into the scratch (Figure 5.3A). In some cases, PAK1$^{-/-}$ BMMs spread and migrated into the scratch at a greater rate than WT BMMs (Figure 5.3B, Movies 5.3 and 5.4). However, tracking of the movies (data not shown) showed this was not reproducible. PAK1, therefore, is not essential in BMMs during scratch assays.

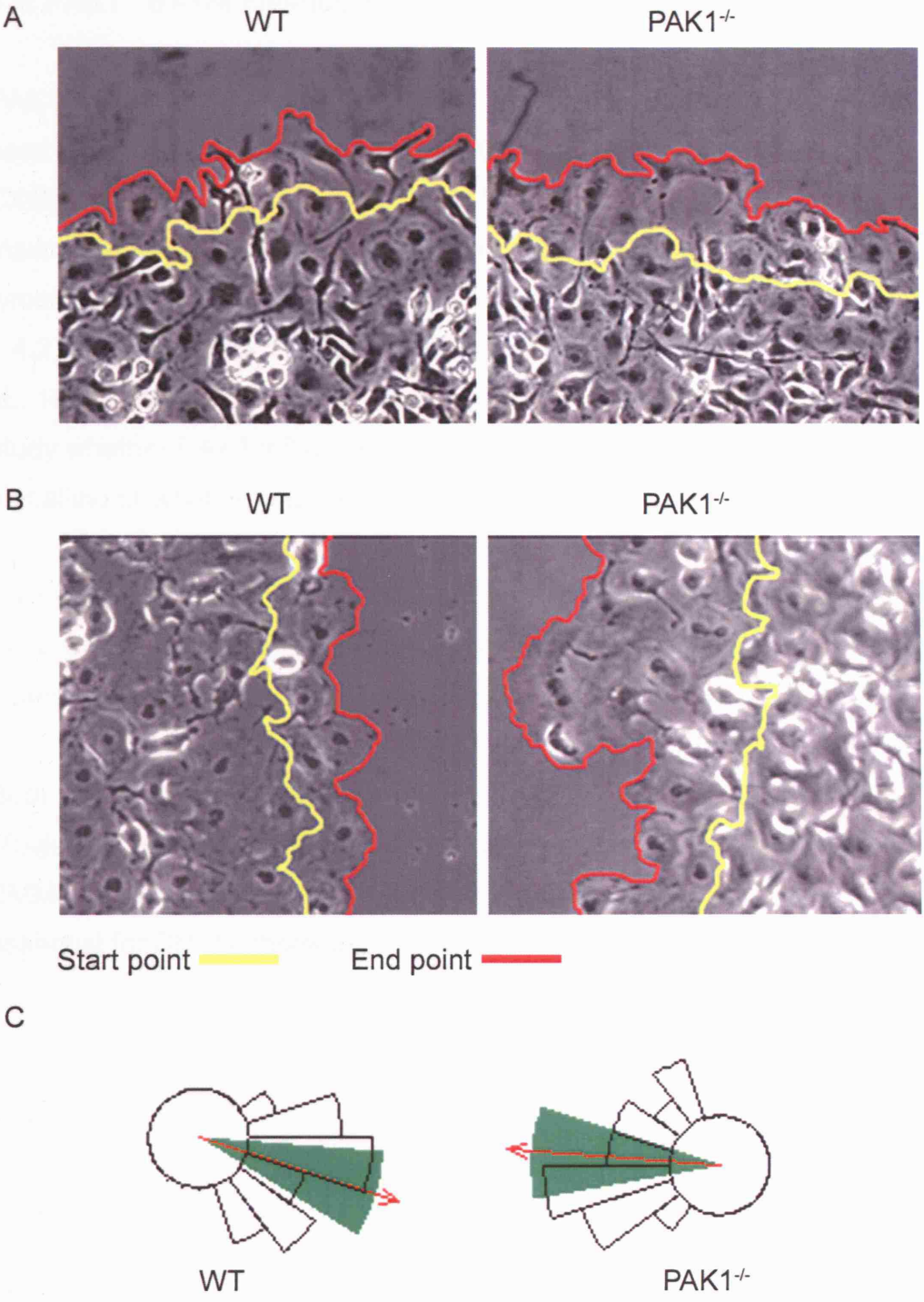



**Figure 5.3:** WT and PAK1$^{-/-}$ BMMs respond similarly to monolayer scratches

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium on tissue culture plastic until a confluent monolayer was formed. (A and B) Scratches were made in the monolayer and timelapse microscopy was used to study migration at the scratch edge (Movies 5.3 and 5.4). Occasionally, PAK1$^{-/-}$ BMMs migrated quicker than WT BMMs (B) but this was not consistently observed. C) WT and PAK1$^{-/-}$ BMMs timelapse movies were tracked using Motion analysis software. Rayleigh plots indicate that WT and PAK1$^{-/-}$ BMMs specifically migrate into the scratch. Results shown are representative of four separate experiments.

### 5.5: PAK1$^{-/-}$ BMMs chemotax towards CSF-1

PAK1 has been previously implicated in chemotaxis, although both reports used G protein-coupled receptor (GPCR)-stimulating chemokines, C5a and CXCL-1 respectively (Li et al., 2003; Wang et al., 2002). CSF-1 signals to macrophages through binding to its receptor, CSF-1R, which is a receptor tyrosine kinase (RTK) (see review (Pixley and Stanley, 2004) and Chapter 1.4.2). Macrophages have been shown to chemotax towards CSF-1 (Allen et al., 1998; Jones et al., 2003; Wells et al., 2004) and can therefore be used to study whether PAK1 influences macrophage chemotaxis downstream of RTK signalling or whether it is a GPCR-specific response.

To investigate this process, BMMs adhered to glass coverslips were exposed to a CSF-1 gradient on a Dunn chamber in which the BMMs could be observed by timelapse microscopy (Figure 2.3).

Both WT and PAK1$^{-/-}$ BMMs migrated up a CSF-1 gradient (Figure 5.4, Movie 5.5 and 5.6). Tracking of the cells showed that the WT and PAK1$^{-/-}$ BMMs migrated towards the source of the CSF-1 indicating that PAK1 is not essential for BMMs chemotaxis towards CSF-1.

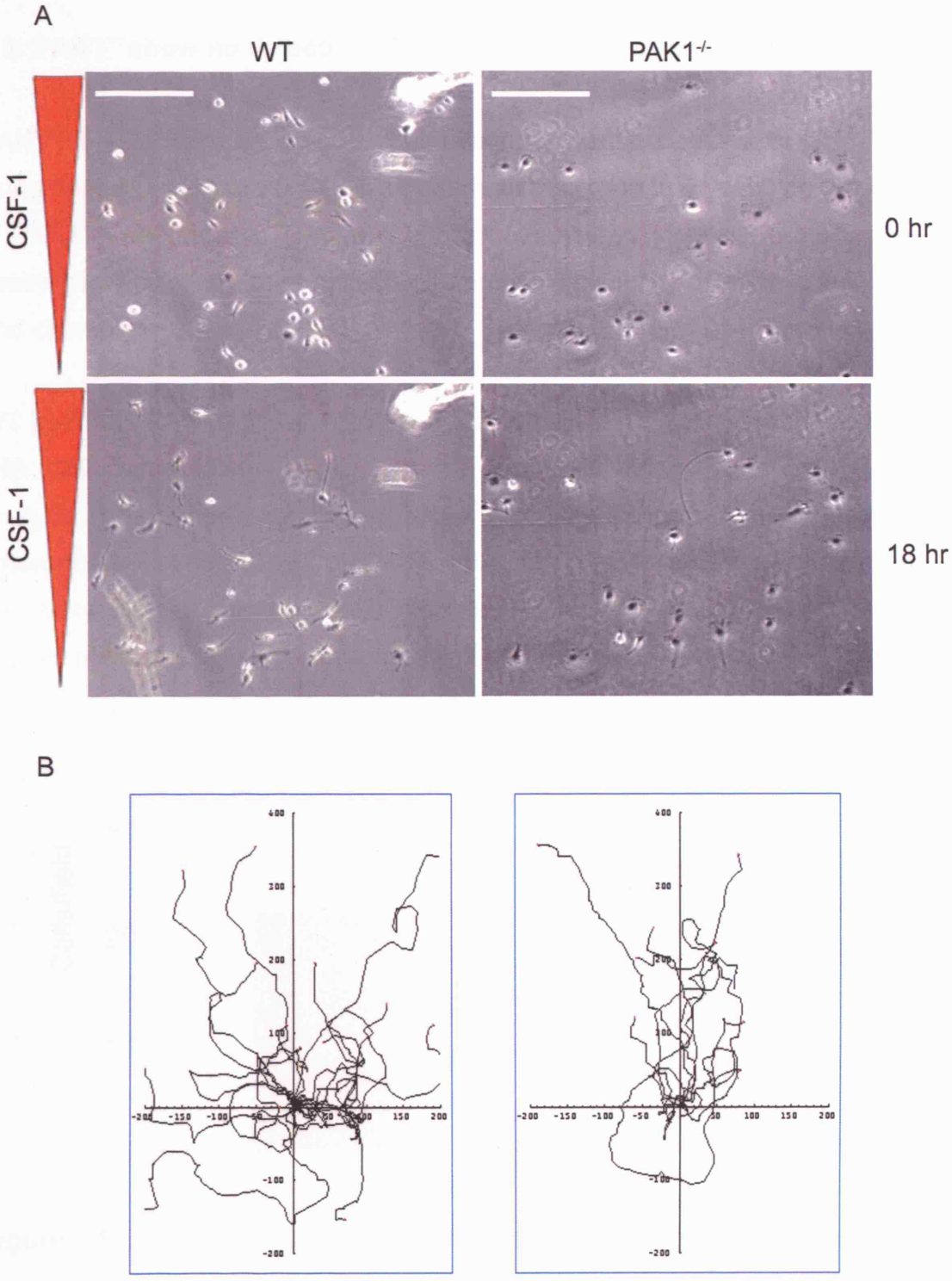



**Figure 5.4:** PAK1$^{-/-}$ BMMs chemotax towards CSF-1

WT and PAK1$^{-/-}$ BMMs were cultured on glass coverslips in growth medium for 6 hours before being starved of CSF-1 for 16 hours. A) Chemotaxis towards 33 ng/ml CSF-1 was evaluated using Dunn chambers. Timelapse microscopy movies were recorded over 18 hours (1 frame/10 min), bars = 100 μm B) BMMs in the Dunn chamber movies were tracked using Motion Analysis. Plot shows the paths migrated by BMMs with the CSF-1 source at the top. n = 15 (WT) and 9 (PAK1$^{-/-}$), results are representative of two separate experiments.

### 5.6: PAK1$^{-/-}$ show no defects in Transwell chemotaxis assays

PAK1$^{-/-}$ BMMs were capable of chemotaxing towards a source of CSF-1 in Dunn chambers (Figure 5.4). As an alternative method to investigate the role of PAK1 in chemotaxis, Transwell chemotaxis assays were performed. This assay has frequently been used to determine the ability of cells to migrate and chemotax (Shi et al., 2003).

WT and PAK1$^{-/-}$ BMMs were plated in starve medium on to a Transwell filter with CSF-1-containing medium in the chamber below. After 24 hours, analysis of BMMs chemotaxis towards CSF-1 showed no significant difference between the WT and PAK1$^{-/-}$ BMMs genotypes (Figure 5.5). The variability in cell numbers observed in each field counted may make identification of subtle differences difficult to observe.

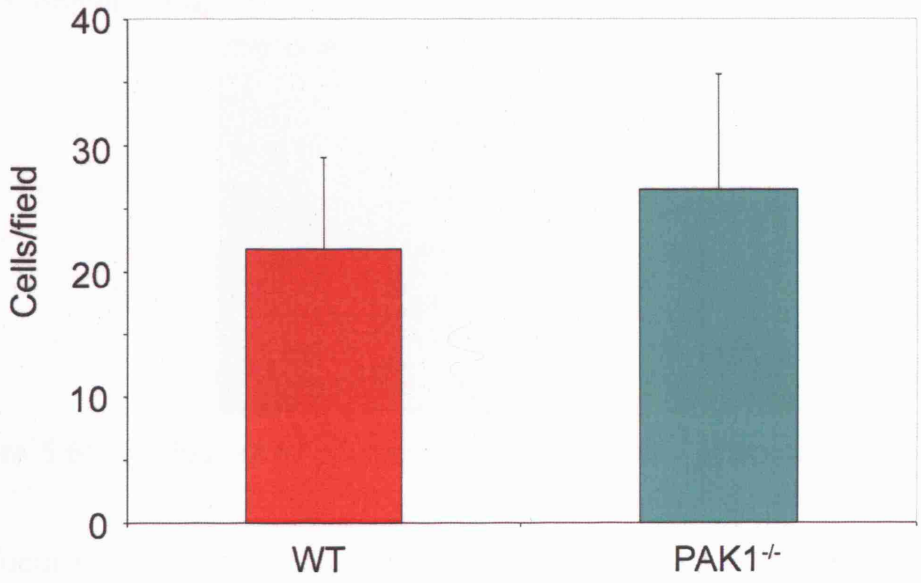



**Figure 5.5:** PAK1$^{-/-}$ BMMs show no defect in CSF-1-induced chemotaxis

WT and PAK1$^{-/-}$ BMM were cultured in growth medium before being suspended in starve medium. BMM medium containing 33 ng/ml CSF-1 was added to the bottom chamber of a 5 $\mu$m pore Transwell (Corning Inc.) and 1x10$^5$ cells in starve medium were plated into the upper chamber. Cells were incubated for 24 hr before fixation and staining with Quick-Diff (Reagena). Results are mean number of cells in each field of view +/- s.e.m. from 3

independent experiments performed in triplicate. Student's t-test analysis indicated no significant difference in chemotaxis between phenotypes.

### 5.7: PAK1 may affect Rho signalling in BMMs

A small number of PAK1$^{-/-}$ BMMs were observed to have an elongated phenotype in the timelapse microscopy movies (Figure 5.7A). The elongated PAK1$^{-/-}$ BMMs were considerably more elongated than any observed in the WT BMMs and were reminiscent of BMMs after treatment with the Rho inhibitor C3-transferase (Allen et al., 1997) and Figure 5.6) and the ROCK inhibitor Y-27632 (Dr. Ann Wheeler, personal communication). Inhibition of Rho signalling produces an elongated phenotype because of a reduction in cell contractility. It is possible that PAK1$^{-/-}$ BMMs also show this phenotype because PAK1 is involved in the regulation of MLC phosphorylation, as shown earlier (Chapter 1.3.3.1 and Figure 4.6).

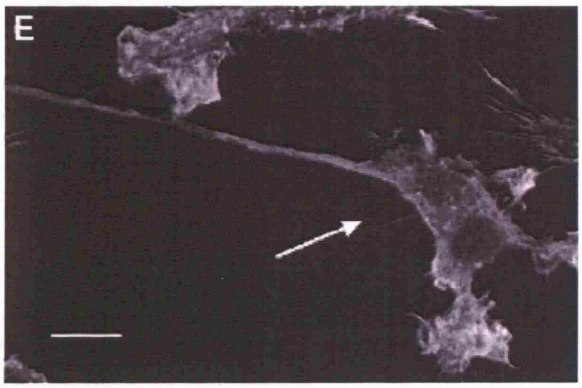



**Figure 5.6:** Inhibition of RhoA produces an elongated phenotype

Confocal image of a Bac1.2F5 cell treated with 2 $\mu$g/ml C3-transferase for 18 hours and stained with TRITC-phalloidin. Bar represents 10 $\mu$m. Image taken from (Allen et al., 1997).

Data supporting a role for PAK1 in RhoA signalling have been reported. PAK1 can phosphorylate and inhibit the RhoA-specific GEFs, NET1 in HEK 293 cells (Alberts et al., 2005) and GEF-H1 in Jurkat T-lymphoblast cells (Zenke et al., 2004). GEF-H1 is a microtubule-bound GEF which is activated upon changes in microtubule dynamics (Krendel et al., 2002). This raises the

possibility that PAK1 is capable of further regulating GEF-H1 through its effector Op18. This implicates PAK1 in RhoA activity regulation and might explain the elongated phenotype seen in some PAK1$^{-/-}$ BMMs.

Before PAK1 regulation of the GEFs was investigated, it was first necessary to determine whether they are expressed in BMMs. WT and PAK1$^{-/-}$ BMM cell lysates were resolved by SDS-PAGE and western blotted to determine the expression profile of the GEFs. A GEF-H1 antibody showed that whilst MDCK cells, HUVECs and NIH-3T3 cells express GEF-H1, BMMs do not (Figure 5.7B). A NET1-specific antibody showed that NIH-3T3 cells express NET1 whilst HT29, DU145, PC3 and WT BMMs do not (Figure 5.7C). These results therefore indicate that whilst PAK1 may regulate RhoA activity in migrating BMMs, it is not via the GEFs NET1 or GEF-H1.

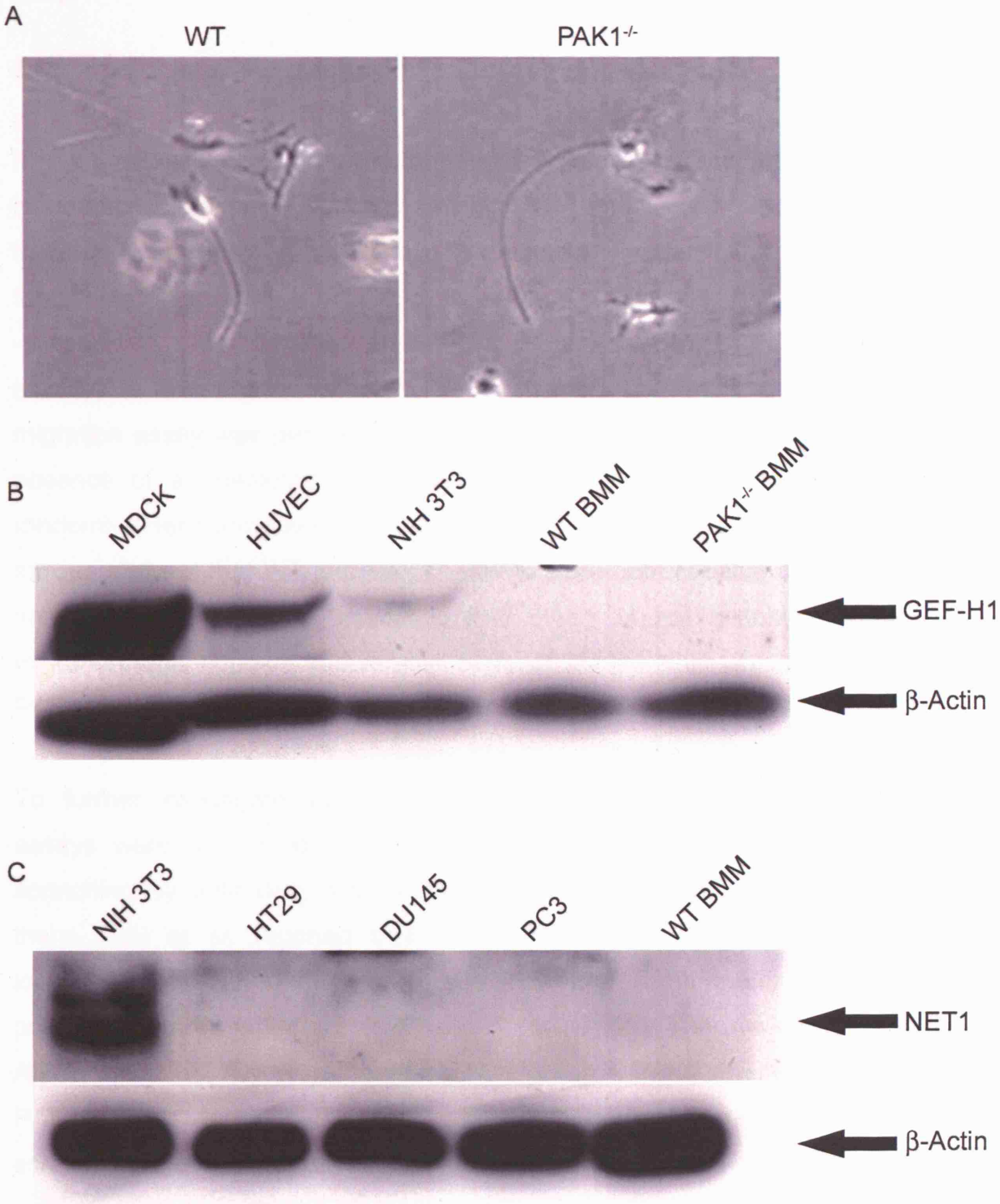



**Figure 5.7:** PAK1 may affect tail retraction in BMMs but not via the RhoGEFs GEF-H1 or NET1.

A) Images taken from Figure 5.5 of WT and PAK1$^{-/-}$ BMMs showing examples of a long tail phenotype. Expression of (B) GEF-H1 or (C) NET1 was determined using western blotting of a range of cell lysates made from cells in growth medium. DU145 = human prostate carcinoma cells, HT29 = human colon cancer cells, HUVEC = human umbilical vein endothelial cells, MDCK = Canine kidney cells, NIH 3T3 = murine fibroblasts, PC3 = human prostate cancer cells. β-actin was used as a loading control and results are representative of two separate experiments.

## 5.8: Conclusions and discussion

It has previously been reported that PAK1 regulates cell migration and the chemotactic response (Chapter 1.3.4). WT and PAK1$^{-/-}$ BMMs were, therefore, used in migration assays to establish whether PAK1 is essential for cell motility.

Initially, to investigate whether PAK1 affected cell motility, a random migration assay was performed. Cells in tissue culture often migrate in the absence of a chemotactic gradient and so are described as migrating randomly (Hermanowski-Vosatka et al., 1999; Kuo et al., 2006). Timelapse movies showed that WT and PAK1$^{-/-}$ BMMs were both capable of polarising and randomly migrating, indicating that PAK1 is not essential for cell migration. This appears to contradict some of the literature which suggested PAK1 was required for migration in other cell types (Chapter 1.3.4).

To further investigate whether PAK1 influences cell migration, scratch assays were performed. WT and PAK1$^{-/-}$ BMM monolayers respond to scratching by extending lamellipodia into the scratch and migrating in to there. Cau *et al.* reported that PAK was required for scratch-induced localisation of $\beta$-PIX to the leading edge promoting membrane extension in a polarised manner in fibroblasts (Cau and Hall, 2005). The use of the PAK1 AID in the study, however, means only the group A PAKs can be implicated. PAK1$^{-/-}$ BMMs were capable of extending lamellipodia and migrating into the scratch suggesting protrusion polarisation was unaffected by PAK1. This discrepancy could be due to cell type differences or redundancy within the PAK family. Analysis of scratch movies indicated that PAK1$^{-/-}$ BMMs migration speed was variable, some times being faster than WT BMMs and in other occasions slower. It is unclear what factor is affecting this change in migration speed but it could potentially be due to slight differences in confluency or scratch width.

PAK1 has been implicated in membrane protrusion dynamics previously. PAK1 was shown to produce novel neurite-like structures in an ACK-Rac-

PAK1-PIX dependent-manner (Heckman et al., 2004) whilst membrane targeting of PAK1 in PC12 cells induced neurites (Daniels et al., 1998). Interestingly, a role for PAK1 has also been identified in spreading upon adhesion, signalling via the ERK pathway (Eblen et al., 2004; Pullikuth et al., 2005; Sundberg-Smith et al., 2005) and Chapter 6). PAK1 may therefore be required for regulation of membrane dynamics during processes such as spreading and lamellipodial extension rather than being involved in extension itself.

A role for PAK1 in regulation of membrane dynamics and membrane polarity could affect migratory persistence. Activation of LIMK by PAK1 was shown to inhibit cofilin activity towards actin filaments, increasing actin stability. Regulation of cofilin activity was also shown to be essential for migration persistence (see review (Bailly and Jones, 2003)) and chemotaxis (Nishita et al., 2005) through depolymerisation of actin at the leading edge (Dawe et al., 2003). The displacement data determined from tracking the random migration movies indicated that the WT and PAK1$^{-/-}$ BMMs migrated a similar distance. However, to establish with more certainty whether loss of PAK1 affects BMM migration persistence, the persistence was determined. Analysis indicated no significant difference between WT and PAK1$^{-/-}$ BMMs during random migration. This suggested that PAK1 is not required for the persistence of migration despite regulating LIMK activity after CSF-1 stimulation (Figure 4.5).

The Rho GTPases Rac1 and Cdc42 are frequently implicated in chemotaxis making PAK1 a likely partner in this process. Whilst Rac is required for the formation of a leading edge, Cdc42 determines the location and stability of the lamellipodia (Srinivasan et al., 2003). Much of the reported results implicate PAK1 signalling in regulation of chemotaxis and directionality rather than migration itself. Use of the PAK AID and a kinase dead PAK1 mutant indicated a requirement for PAK1 in LPA-induced chemotaxis (Jung et al., 2004) and PAK1 was also implicated in chemotaxis towards the chemokine C5a (Li et al., 2003). This suggests that PAK1 is not involved directly in the process of cell migration but may act as a downstream regulator of Cdc42 in

the process of chemotaxis (Allen et al., 1998; Li et al., 2003). However, other possible mechanisms for chemotaxis exist such as the binding of active Rac or Cdc42 to IQGAP1 and APC (Noritake et al., 2005). The adhesion protein L1 has also been shown to stimulate PAK1 during chemotaxis via Vav2 and Rac1. Stimulation of PAK1 in L1-transfected HEK293 cells promoted cell migration via ERK activation (Schmid et al., 2004).

PAK1 may therefore be involved in Cdc42-mediated stabilisation of lamellipodium rather than Rac-induced formation. The previously reported role of PAK1 in chemotaxis may be due to loss of lamellipodial stability or localisation. Cdc42 has been shown to be involved in macrophage chemotaxis towards CSF-1 (Allen et al., 1998), and so a potential role for PAK1 in this process was investigated using Dunn chambers.

Using CSF-1 as a chemo-attractant, it was possible to film and track WT and $PAK1^{-/-}$ BMMs chemotaxing. PAK1 was not required for BMM chemotaxis towards CSF-1 although the use of Dunn chambers proved inconsistent with BMMs frequently not migrating or chemotaxing. To establish further whether PAK1 is required for chemotaxis, a Transwell assay was performed. Pradip *et al.* incubated BMMs for 4 hours to investigate haptotaxis towards a number of extracellular matrix proteins (Pradip et al., 2003), however, analysis of BMMs after 6 hours in a CSF-1 gradient showed the presence of few migrated cells. A longer incubation period was therefore used (Wheeler et al., 2006). $PAK1^{-/-}$ BMMs were capable of chemotaxis towards CSF-1 and there was no significant difference in chemotactic response between WT and $PAK1^{-/-}$ BMMs.

These data indicate that PAK1 is not essential for BMMs migration or chemotaxis towards CSF-1. PAK2 was activated during BMMs chemotaxis towards RANTES (Weiss-Haljiti et al., 2004), suggesting a possible role for PAK2, and may therefore also be responsible for CSF-1-induced chemotaxis. The reports implicating PAK1 in chemotaxis used transfected HEK-293 cells (Wang et al., 2002) and the macrophage cell line RAW274 (Li et al., 2003) which could use slightly different signalling pathways to primary

BMMs. Therefore, it is possible that PAK2 is responsible for the regulation of chemotaxis in BMMs. It is feasible, however, that the PAK family has a level of redundancy in these pathways. PAK2 may be capable of replacing PAK1, masking any defects in $PAK1^{-/-}$ BMMs. Use of PAK2-specific RNAi or the PAK AID would help elucidate whether PAK2 is utilised in $PAK1^{-/-}$ BMM chemotaxis towards CSF-1. PAK2 RNAi in WT BMM would also allow determination of whether PAK2 was the main target in CSF-1-induced chemotaxis or whether a role is due to redundancy within the PAK family.

Another possible explanation is the type of chemo-attractant. Previous studies reporting a role for PAK in chemotaxis have used chemokines that signal through GPCRs. CSF-1 is different as its receptor is an RTK and may induce similar responses through different mechanisms. GPCRs and RTKs activate different downstream effectors, such as different members of the PI3-K family. RANTES stimulation of its GPCR in BMMs requires the PI3-K$\gamma$ isoform (Weiss-Haljiti et al., 2004) whereas CSF-1 stimulation of its RTK in BMMs required PI3-K$\delta$ and PI3-K$\beta$ for cell migration (Vanhaesebroeck et al., 1999). PI3-K and PTEN regulation of $PIP_3$ levels is essential for chemotaxis (see review (Merlot and Firtel, 2003)) and so it is possible that targeting of different PI3-K isoforms by the receptors activates alternative signalling mechanisms for the induction of chemotaxis. It is therefore possible that GPCR-induced chemotaxis signals via PI3-K$\gamma$ and PAK whilst CSF-1 utilises PI3-K$\delta$ and PI3-K$\beta$ to activate other pathways implicated in chemotaxis such as Cdc42's interaction with IQGAP1 and APC (Noritake et al., 2005) or through Cdc42 and WASp (Zicha et al., 1998). Separate requirements for GPCR and RTK mediated chemotaxis could be studied through use of a GPCR-stimulating chemokine in $PAK1^{-/-}$ BMMs. Preliminary results using monocyte chemotactic protein-1 (MCP-1) in Transwell chemotaxis assays showed no significant changes between WT and $PAK1^{-/-}$ BMMs suggesting PAK1 is not required for MCP-1-induced chemotaxis either (data not shown). Further investigation with MCP-1 and a chemokine such as RANTES would identify whether PAK1 is required for GPCR-mediated chemotaxis in BMMs. Another possibility is a direct interaction between GPCRs and PAK. Li *et al.* suggested that the PAK1 N-terminus interacts with the $G_{\beta\gamma}$ subunit of

GPCRs. The PAK binding partner $\alpha$-PIX then activates Cdc42 and resultantly PAK1, leading to an induction of polarity and chemotaxis (Li et al., 2003). The inability of RTKs such as CSF-1R to induce PAK1 localisation and activation directly may be responsible for the apparent lack of PAK1 function in CSF-1-induced chemotaxis.

PAK1$^{-/-}$ BMMs are occasionally observed to have an extremely elongated phenotype. This appears similar to the effect observed in cells treated with the Rho GTPase inhibitor C3 transferase and the ROCK inhibitor Y-27632. PAK1 has been shown to inhibit the Rho GEFs, NET1 (Alberts et al., 2005) and GEF-H1 (Zenke et al., 2004). This disruption to RhoA regulation may explain why some PAK1$^{-/-}$ BMMs show a phenotype indicative of reduced cell contractility. However, BMMs express neither NET1 nor GEF-H1, showing that any PAK1 regulation of RhoA in BMMs is not through these GEFs. It is possible that PAK1 may regulate a different Rho GEF, although no others have been identified in the literature and Blast searches of the NET1 and GEF-H1 primary sequences showed no obvious homologues that BMMs might express (data not shown). Another possibility is PAK1 regulates a RhoGDI, proteins that inhibit Rho GTPase targeting to the membrane and activation. PAK1 phosphorylated RhoGDI in 293T cells; however, PAK1 phosphorylation of RhoGDI promoted dissociation of Rac1 but not RhoA (DerMardirossian et al., 2004). No other data have indicated PAK1 as a regulator of RhoGDIs; however, it is possible that PAK1 phosphorylates other RhoGDI isoforms in BMMs such as the haematopoietic cell-specific RhoGDI-$\beta$ isoform (see review (Dovas and Couchman, 2005)). PAK1 may also phosphorylate distinct residues downstream of different cytokines which could affect dissociation of specific Rho GTPases.

It is also possible that PAK1 does not regulate RhoA in this process. The elongated cells may be a result of defective cell contraction, via reduced phosphorylated MLC levels. As was shown, loss of PAK1 in BMMs disrupts MLC regulation (Figure 4.6) which could produce the defect in contraction observed in some PAK1$^{-/-}$ BMMs. The elongated phenotype could potentially also be caused by a reduction in adhesion turnover and a prevention of tail

retraction. PAK1 has been implicated in adhesion turnover and disassembly (Zhao et al., 2000), and so loss of PAK1 could result in increased adhesion at the tail of the cell. Immunofluorescence study of cell adhesions in migrating cells would help determine whether this was the cause.

To summarise, PAK1 is not required for cell polarisation upon adhesion or for random migration. Migratory persistence was also unaffected suggesting PAK1 is not required for lamellipodial localisation or stabilisation during migration. It also indicates that PAK1 is not required for Rac-induced lamellipodial extension, whilst the similarity in migration speeds between WT and $PAK1^{-/-}$ BMMs suggests PAK1 regulation of adhesion turnover is not essential during migration. The results also show that PAK1 is not required for BMM chemotaxis towards CSF-1 and suggest that overall, PAK1 has no clear role in BMM migration. A potential role in tail retraction has been observed in a small number of cells exhibiting an extremely elongated phenotype. This could be caused by changes in adhesion turnover, actomyosin contractility or changes to RhoA activity. However, PAK1 mediated regulation of RhoA appears unlikely as the Rho GEFs, NET1 and GEF-H1, which are involved in PAK-dependent inactivation of RhoA, are not expressed in BMMs.

# Chapter 6: PAK1 regulation of cell spreading.

## 6.1: Introduction

Engagement of integrins leads to firm cell adhesion and links the cytoskeleton to the extracellular matrix (see review (Brakebusch and Fassler, 2003)). Integrin engagement also promotes activation of a large number of signalling pathways, including the Rho GTPases (see review (DeMali et al., 2003)). For example, fibroblast adhesion induces Rac and Cdc42 activation, which is essential for cell spreading (Price et al., 1998), whilst in keratinocytes, the Rho GEF, T-lymphoma invasion and metastasis 1 (Tiam1), is required for $\alpha 3\beta 1$-mediated laminin-5 deposition and spreading (Hamelers et al., 2005). Also, the Rho GEF Vav1 mediates integrin-induced spreading in T-cells (del Pozo et al., 2003). Taken together these results suggest a strong link between integrin engagement, Rho GTPase activity and spreading.

Integrin-induced stimulation of Rho GEFs is mediated by a number of tyrosine kinases. These include the ubiquitously expressed focal adhesion kinase (FAK) and the primarily neuronal and haematopoietic proline-rich tyrosine kinase 2 (Pyk2), which are activated upon integrin engagement (Astier et al., 1997; Richardson and Parsons, 1996). Activation of Pyk2 stimulates Rho and PI3-K (Okigaki et al., 2003), and FAK phosphorylates paxillin kinase linker (PKL) (Brown et al., 2005) and paxillin, which may influence active Rac levels (Lamorte et al., 2003; Valles et al., 2004).

Integrin activation of the Rho GTPases suggests PAK may be a mediator in cell spreading. Indeed, several lines of evidence implicate PAK in cell spreading (Chapter 1.3.5). The PAK-associated protein $\alpha$-PIX influences cell spreading via integrin signalling. Cell attachment promotes Rho GTPase activation through the GEF activity of $\alpha$-PIX and cell spreading via its association to Calpain 4, a process independent of its GEF activity (Rosenberger et al., 2005). Calpain 4 is a regulatory subunit for the calpain proteases which influence spreading through proteolysis of focal adhesion

proteins including paxillin, talin and $\alpha$-actinin (see review (Frame et al., 2002)). PAK has also been directly implicated in platelet spreading through interaction with cortactin (Vidal et al., 2002), a cortical actin binding protein that can interact with the Arp2/3 complex to promote actin polymerisation after engagement of $\alpha_2\beta_1$ integrin on collagen (Suzuki-Inoue et al., 2001). In addition, EphrinA1 has been identified as an inhibitor of integrin-mediated spreading and functions by inactivating the Rac/PAK1 signalling pathway in vascular smooth muscle cells (Deroanne et al., 2003).

These data strongly implicate Rho GTPases and PAK in cell spreading. PAK1$^{-/-}$ BMMs were used to investigate the role PAK1 plays in macrophage spreading upon adhesion.

## Results

### 6.2: PAK1 regulates BMMs spreading upon adhesion

PAK1 has been implicated in the regulation of platelet spreading upon adhesion downstream of Src, PI3-K and Rac (Suzuki-Inoue et al., 2001; Vidal et al., 2002). To investigate a potential role for PAK1 in BMM spreading, WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips, stained for F-actin and analysed using confocal microscopy and MetaMorph software.

PAK1$^{-/-}$ BMMs spread to a greater area than WT cells from 5 minutes onwards (Figure 6.1A). Quantification of multiple cell images confirmed that the PAK1$^{-/-}$ BMMs were more spread (Figure 6.1B) and showed they were more elongated by 15 min (aspect ratio) than WT BMMs (Figure 6.1C).

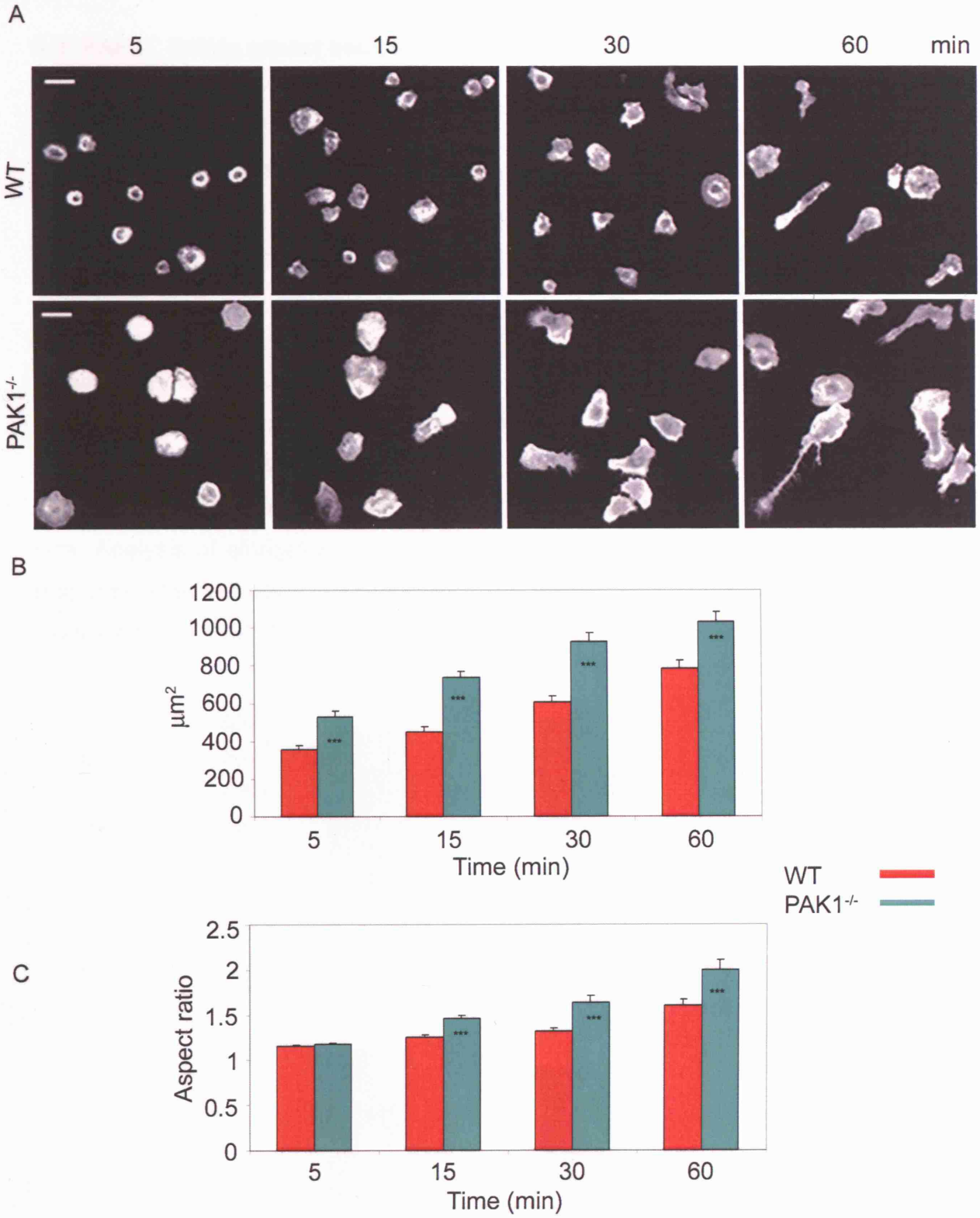



**Figure 6.1:** PAK1$^{-/-}$ BMMs initially spread more rapidly to a larger area and are more elongated on adhesion to glass.

BMMs were cultured in growth medium and then plated onto glass coverslips for between 5 and 60 min in growth medium. A) Confocal images of WT and PAK1$^{-/-}$ BMMs stained for F-actin. Cells were quantitated for (B) cell area and (C) cell aspect using MetaMorph software. Results show the mean area and aspect +/- s.e.m. Student's t-test was used for statistical analysis of the data (*** = $p<0.01$). n= approximately 150 cells for each time point and are from 3 pooled independent experiments.

### 6.3: PAK1$^{-/-}$ BMMs retract back to a normal spread area

Upon adhesion, PAK1$^{-/-}$ BMMs spread to a larger area and became more elongated than WT BMMs. To investigate whether this trend was maintained over time or whether it was a temporary response to adhesion, the spread area and elongation of BMMs 24 hours after adhesion was investigated.

Analysis of confocal images showed no significant difference in spread area between WT and PAK1$^{-/-}$ BMMs after 24 hours. Both sets of macrophages were less spread than 1 hour after adhesion and had retracted to a similar area (Figure 6.2A). WT BMMs were approximately 75% of the spread area observed after 1 hour whilst PAK1$^{-/-}$ BMMs were approximately 60% of the size. Analysis of elongation showed that, in contrast to the early spreading response after adhesion (Figure 6.1C), WT BMMs were significantly more elongated than PAK1$^{-/-}$ BMMs (Figure 6.2B).

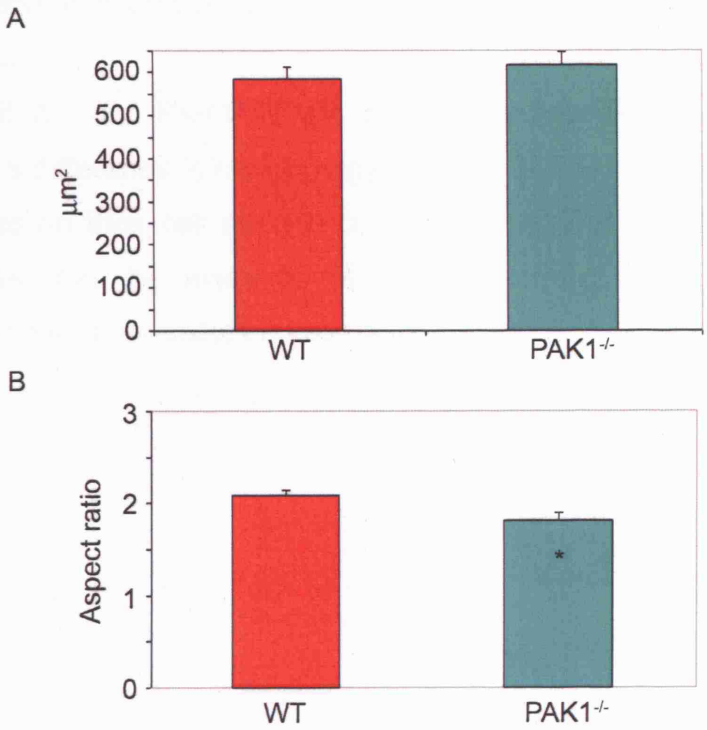



**Figure 6.2:** PAK1$^{-/-}$ BMMs are the same size but less elongated than WT BMMs 24 hours after adhesion to glass.

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium on glass coverslips

for 24hours. BMMs were fixed and stained for F-actin with TRITC-phalloidin. Images were taken with a confocal microscope and cells were analysed for A) cell spread area and B) elongation using MetaMorph. Results show the mean area and aspect +/- s.e.m. Data were analysed using Student's t-test (*= $p<0.05$), n= 132 (WT) and 170 ($PAK1^{-/-}$) pooled from three independent experiments.

### 6.4: $PAK1^{-/-}$ BMMs exhibit a higher number of microspikes on their surface 24 hours after adhesion

$PAK1^{-/-}$ BMMs spread to a larger area than WT BMMs upon adhesion but then retracted back to a spread area similar to WT BMMs. During cell spreading, RhoA activity is reduced via c-Src, FAK and p190RhoGAP (Wakatsuki et al., 2003). Cell retraction, however, is linked to RhoA and ROCK activity (Amano et al., 1996). This suggests that RhoA regulation of cell retraction is unaffected in $PAK1^{-/-}$ BMMs.

Analysis of WT and $PAK1^{-/-}$ BMM images used for Figure 6.2 showed that there was a difference in morphology. $PAK1^{-/-}$ BMMs had a higher number of microspikes on their cell surface compared to WT BMMs (Figure 6.3). The microspikes may be evidence of retraction from a larger spread area although some of the spikes could be filopodia.

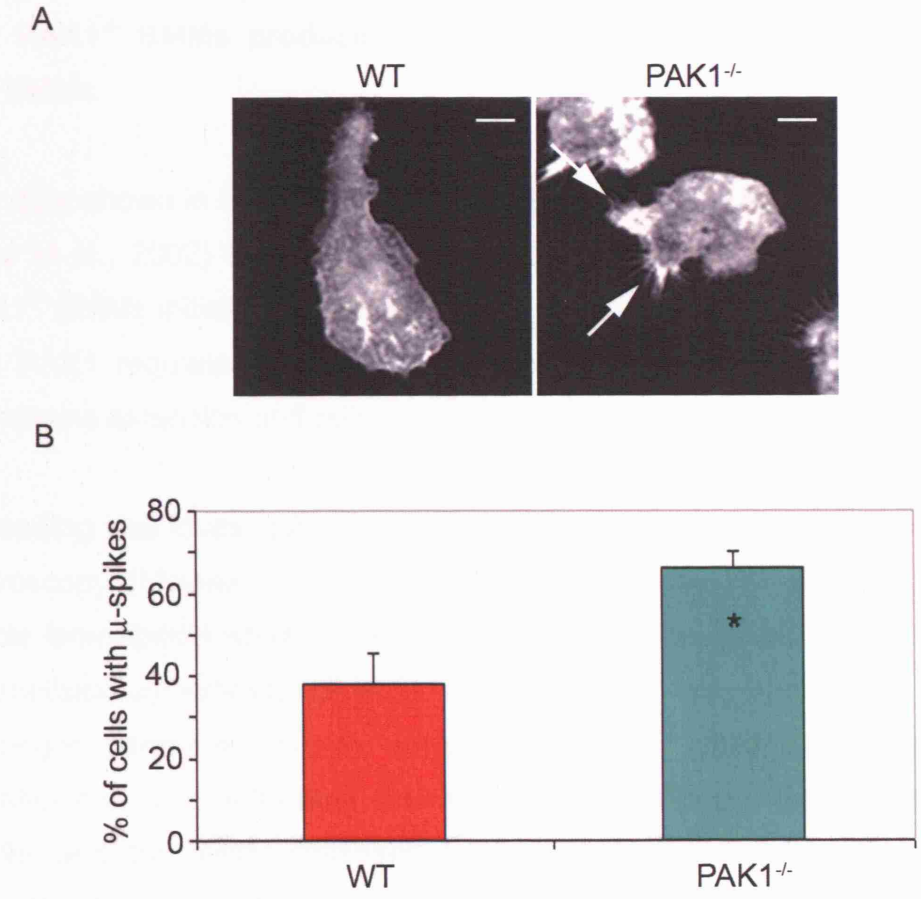


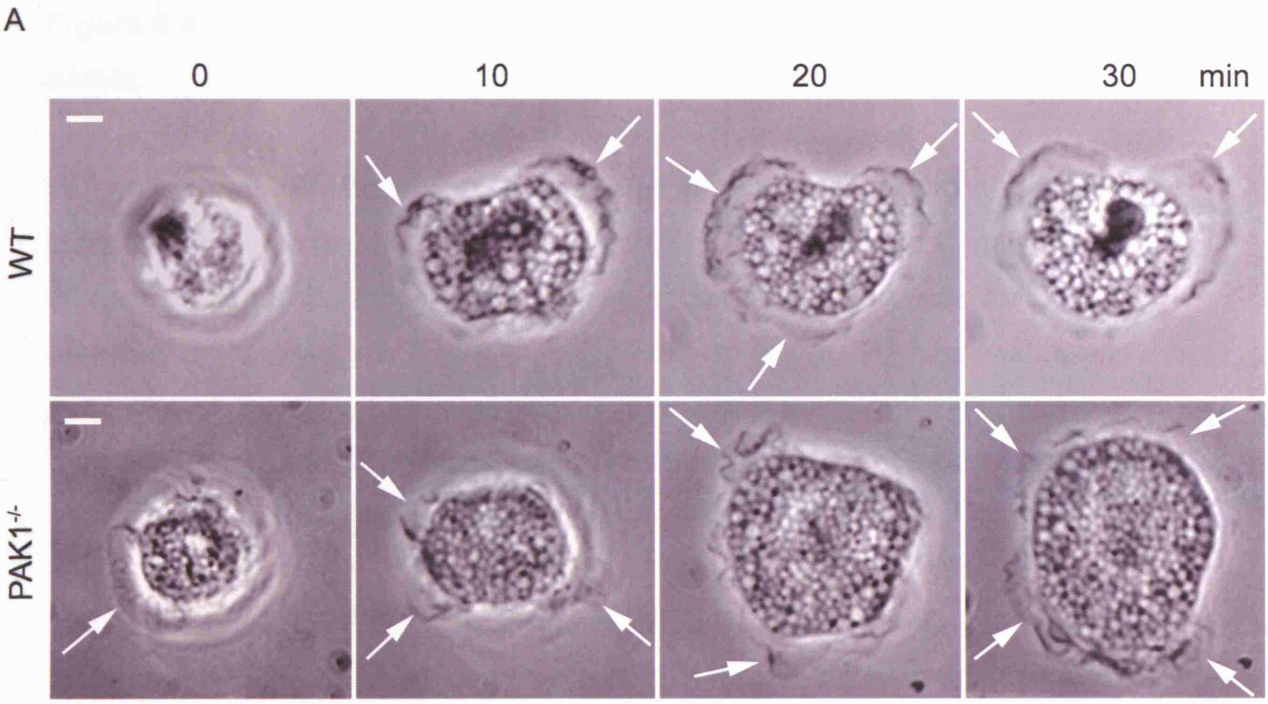

**Figure 6.3:** A greater number of PAK1$^{-/-}$ cells have microspikes.

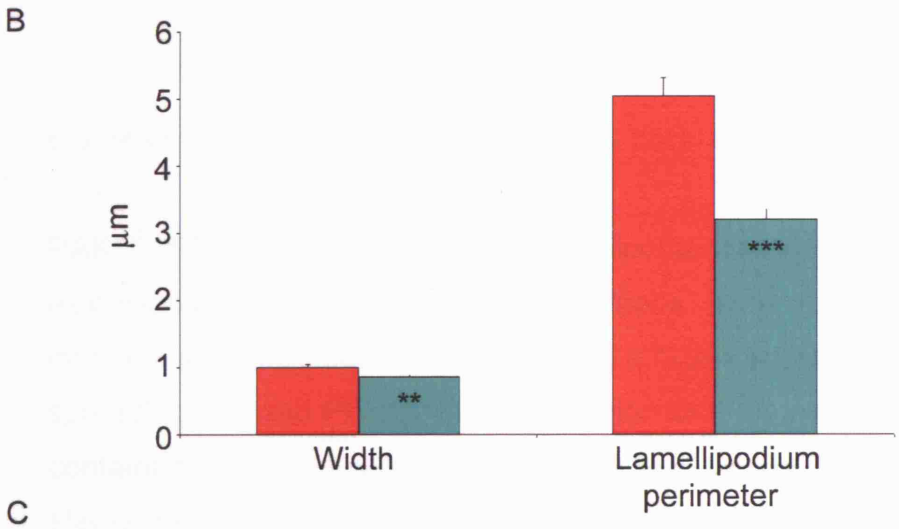
WT and PAK1$^{-/-}$ BMMs were cultured in growth medium on glass coverslips for 24 hours. BMMs were fixed and stained for F-actin with TRITC-phalloidin. A) Images of BMMs were acquired using confocal microscopy. B) BMMs were scored for the presence or absence of microspikes on their surface. Results show percentage of WT and PAK1$^{-/-}$ BMMs scored for having microspikes +/- s.e.m. Student's t-test was used to analyse data, (*= $p<0.05$), n=132 (WT) and 170 (PAK1$^{-/-}$) pooled from 3 independent experiments. Bar = 10µm.

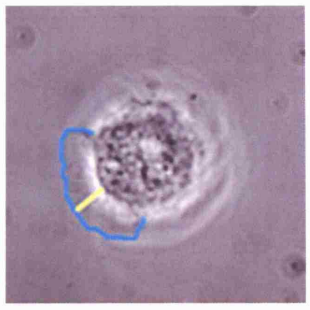
## 6.5: PAK1$^{-/-}$ BMMs produce more lamellipodia during spreading than WT BMMs

The data shown in Figure 6.1 and in the literature (Suzuki-Inoue et al., 2001; Vidal et al., 2002) implicate PAK1 in the process of cell spreading. Since PAK1$^{-/-}$ BMMs initially spread to a greater area than WT BMMs, it is possible that PAK1 regulates early spreading responses and controls the rate of membrane extension and adhesion.

Spreading was investigated in WT and PAK1$^{-/-}$ BMMs using timelapse video microscopy (Movies 6.1 and 6.2). Upon adhesion, WT BMMs produced stable lamellipodia which gradually extended increasing the spread area. As a lamellipodium extended, it encompassed a larger area of the BMM and had a longer perimeter (Figure 6.4A). The PAK1$^{-/-}$ BMMs also extended lamellipodia upon adhesion, however, the lamellipodia formed were less stable and frequently collapsed back into the cell body (Figure 6.4A). Quantification using MetaMorph indicated that the lamellipodia in PAK1$^{-/-}$ BMMs extended a similar, but significantly shorter, distance than WT BMMs, and had a smaller perimeter and therefore surrounded a smaller percentage of the cell border (Figure 6.4B). In addition, PAK1$^{-/-}$ BMMs consistently produced a greater number of lamellipodia than WT BMMs (Figure 6.4C).


A
0
10
20
30
min
WT
PAK1-/-


B
µm
6
5
4
3
2
1
0
**
***
Width
Lamellipodium perimeter


Width
Perimeter
WT
PAK1-/-

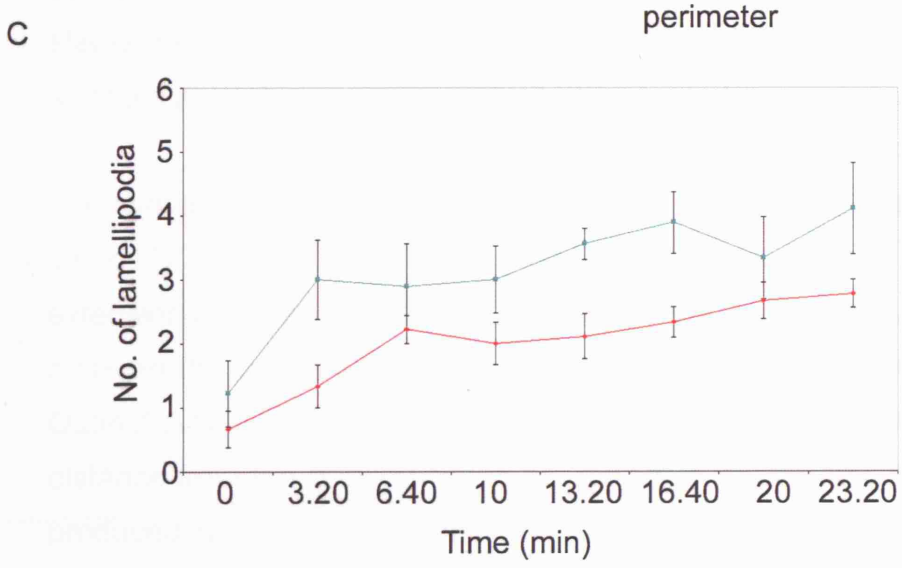

C
No. of lamellipodia
6
5
4
3
2
1
0
0
3.20
6.40
10
13.20
16.40
20
23.20
Time (min)

**Figure 6.4:** PAK1$^{-/-}$ BMMs have more lamellipodia during spreading than WT BMMs.

WT and PAK1$^{-/-}$ BMMs were suspended in growth medium and plated onto glass bottom microwell dishes (MatTek Corp.). A) Timelapse microscopy images of BMM spreading, 1 frame was acquired every 10 sec for 30 min after adhesion (Movies 6.1 and 6.2). Bars = 1 $\mu$m. B) The width and perimeter of the lamellipodia produced during spreading were measured at their peak size and quantitated using MetaMorph. Student's t-test analysis showed PAK1$^{-/-}$ BMM lamellipodia extend a significantly shorter distance (** = $p<0.01$) and have a smaller perimeter (*** = <0.001) than WT BMM, n = 145 WT, n = 225 PAK1$^{-/-}$ lamellipodia, data pooled from 6 independent experiments. C) The number of lamellipodia produced over 23 min were quantified using MetaMorph. Images and data are representative of 9 cells from 6 independent experiments for each genotype.

### 6.6: PAK1$^{-/-}$ BMM lamellipodia are less persistent than in WT BMMs

PAK1$^{-/-}$ BMMs produced more lamellipodia than WT BMMs but these extensions were less stable. Kymographs were made to visualise the increase in lamellipodial collapse. Using a representative timelapse movie of spreading WT and PAK1$^{-/-}$ BMMs, a region of the movie was selected which contained an area of lamellipodium extension (Figure 6.5A). The kymograph stacks the selected region from each frame of the movie next to previous one so changes in that region can be observed with time.

The kymographs showed that in WT BMMs, the lamellipodium extended out smoothly before it plateaued and ruffled with slight retractions and extensions (Figure 6.5B). PAK1$^{-/-}$ BMMs lamellipodia, however, showed a more erratic extension with frequent retractions and collapses (Figure 6.5B). Quantification showed that PAK1$^{-/-}$ BMM lamellipodia extended a similar distance from the cell body as in WT BMMs (Figure 6.4B), but they were not produced in as smooth or controlled a manner.

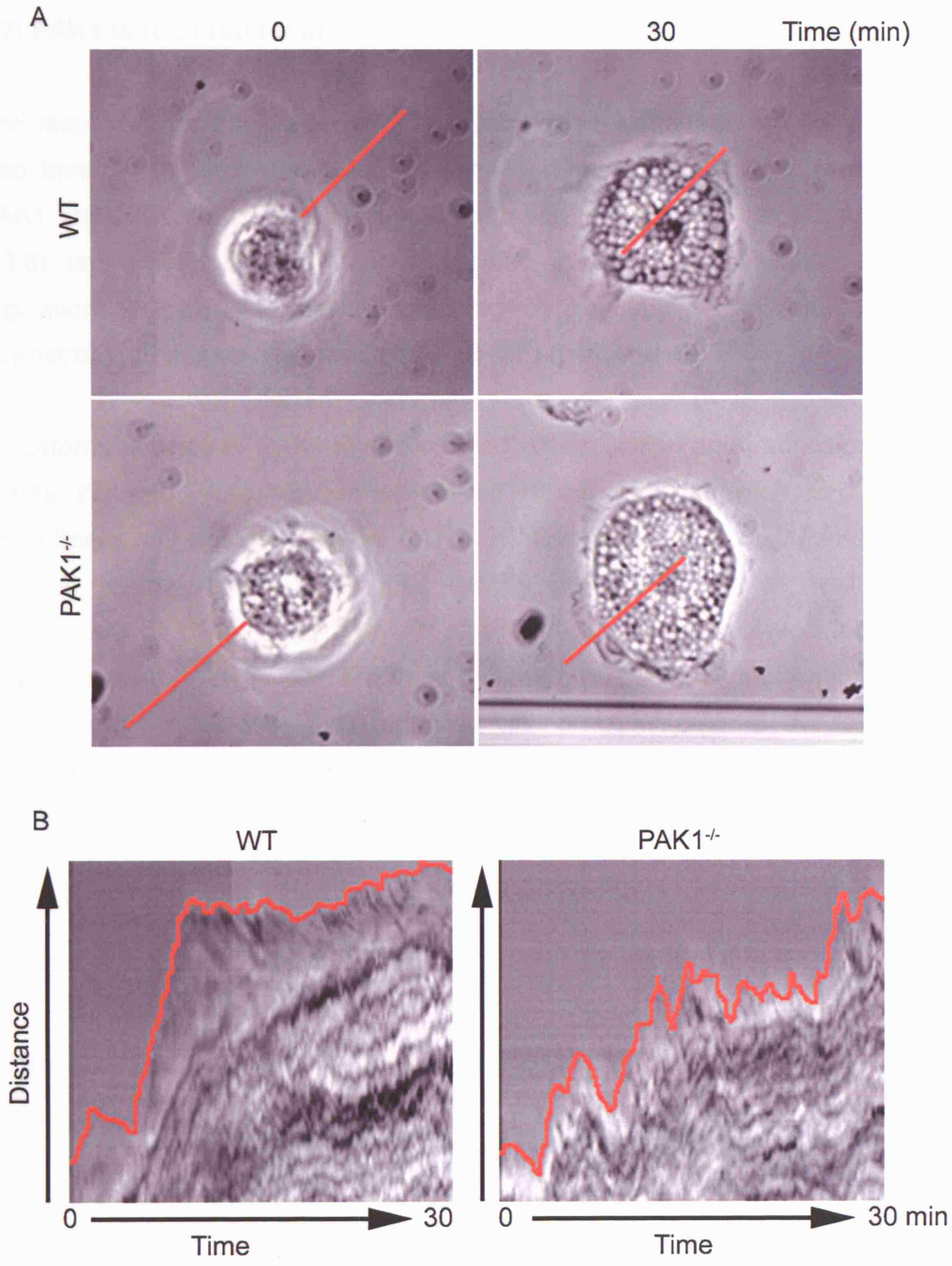



**Figure 6.5:** PAK1$^{-/-}$ BMM produce less persistent lamellipodia when spreading

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium, suspended and then plated onto a glass coverslip-bottomed dish in growth medium. A) Time-lapse microscopy images of WT and PAK1$^{-/-}$ BMMs adhering and spreading. The red line indicates the region measured for production of a kymograph. B) Each kymograph was produced using MetaMorph software.

### 6.7: PAK1 is required for maximal ERK phosphorylation upon adhesion

The results so far implicate PAK1 in macrophage spreading, but PAK1 is also involved in regulating MAPK activation (Figure 4.2). A link between PAK1 and ERK during spreading has been suggested previously (Chapter 1.3.6) and ERK has also been implicated in the phosphorylation and regulation of paxillin-mediated spreading by regulating FAK and Rac downstream of hepatocyte growth factor (HGF) (Ishibe et al., 2004).

To determine whether PAK1 is involved in ERK activation upon adhesion in BMMs, WT and PAK1$^{-/-}$ BMMs were lysed 10 min after adhesion. ERK1/2 phosphorylation was reduced in PAK1$^{-/-}$ BMMs suggesting that PAK1 is involved in the regulation of ERK activity upon adhesion as well as downstream of CSF-1. However, no clear changes were observed in the phosphorylated levels of p38, MEK1 or Pyk2 (Figure 6.6A). Quantification by densitometry confirmed the reduction in phosphorylated-ERK levels compared to total ERK levels (Figure 6.6B).

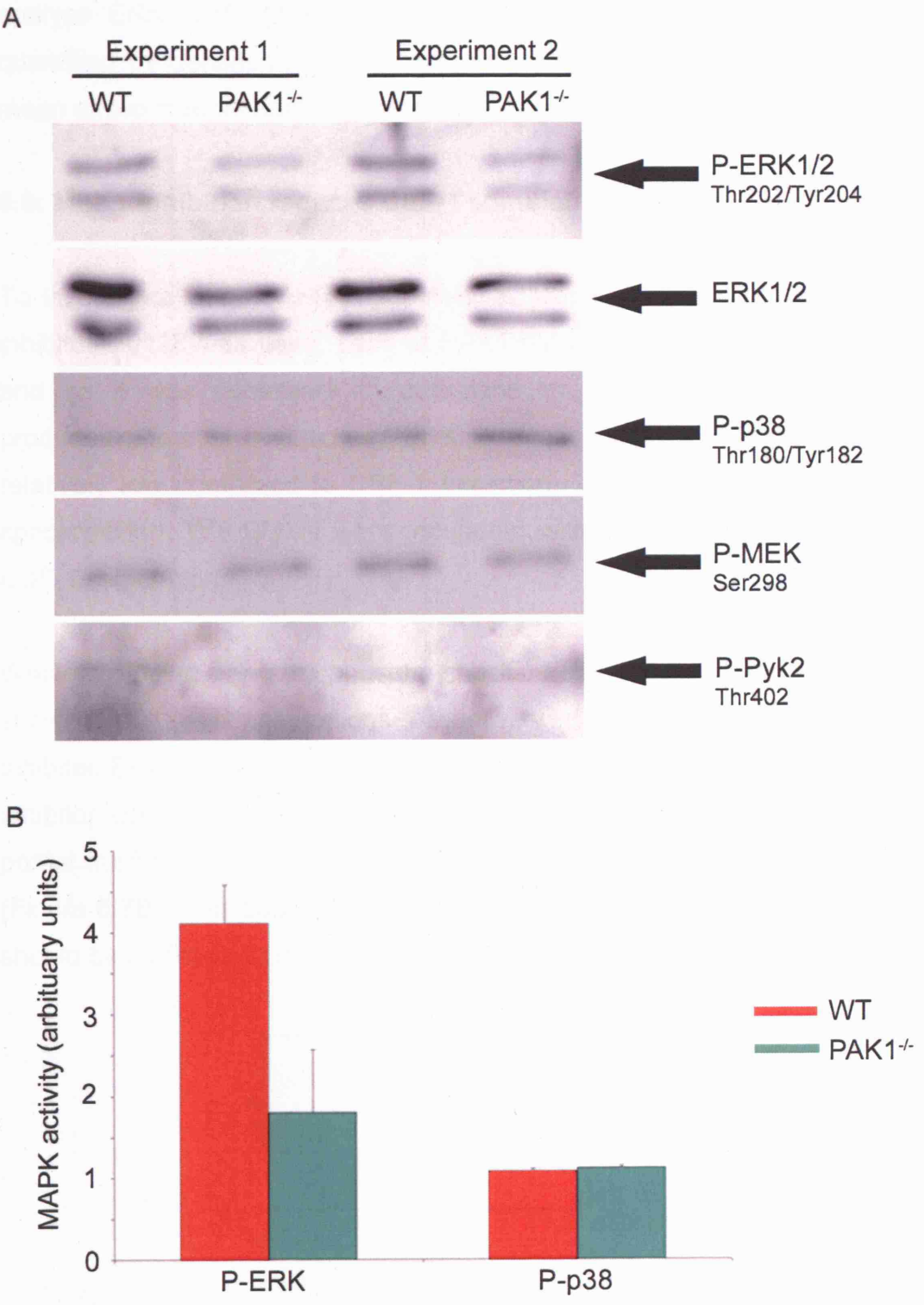



**Figure 6.6:** ERK activation upon adhesion is reduced in PAK1$^{-/-}$ BMMs.

WT and PAK1$^{-/-}$ BMMs cultured in growth medium were suspended and plated onto tissue culture plastic in growth medium for 10 min and then lysed. A) Proteins were separated by SDS-PAGE and western blotted to

analyse ERK, p38, MEK1 and Pyk2 activation. B) Western blots were quantified by densitometry and normalised to ERK levels. Results are the mean of two independent experiments +/- s.d.

### 6.8: MEK1 inhibition reduces CSF-1 stimulation of ERK1/2

To investigate whether ERK1/2 activity is required for spreading, the MEK inhibitor U0126 was used. Loss of PAK1 did not totally inhibit ERK activity and so it was necessary to determine an inhibitor concentration that produced a partial inhibition of ERK. Activation of ERK upon adhesion is relatively low compared to CSF-1 induction. To determine an appropriate concentration, WT BMMs were incubated with the U0126 inhibitor before CSF-1 stimulation.

Western blotting using the phospho-specific ERK1/2 Thr202/Tyr204 antibody showed U0126 concentrations of between 5 and 50 $\mu$g/ml completely inhibited ERK activation upon CSF-1 stimulation (Figure 6.7A). Testing lower inhibitor concentrations showed that 0.5 and 1 $\mu$g/ml U0126 produced a partial inhibition of ERK activity, similar to that observed in PAK1$^{-/-}$ BMMs (Figure 6.7B). This suggests that use of the MEK inhibitor U0126 at 1 $\mu$g/ml should be sufficient to replicate loss of PAK1.

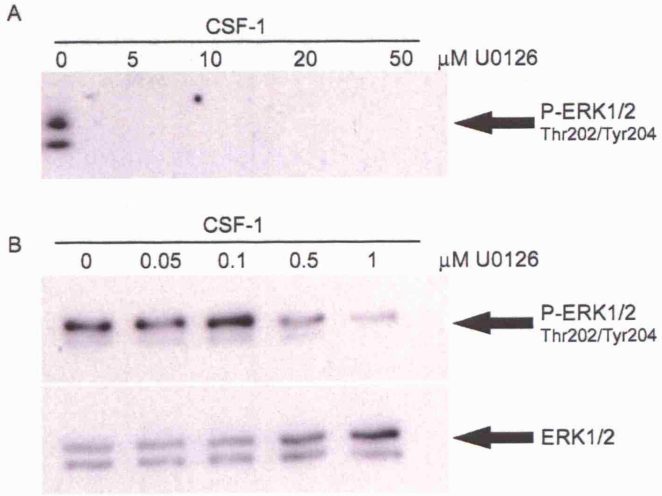



**Figure 6.7**: The MEK inhibitor, U0126 inhibits CSF-1 stimulation of ERK phosphorylation in WT BMMs

WT BMM starved of CSF-1 overnight, were incubated with (A) 0 to 50 µM or (B) 0 to 1 µM MEK inhibitor U0126 for 1 hour. BMMs were stimulated with CSF-1 (33 ng/ml) for 5 min before cell lysis. Samples were western blotted to analyse P-ERK levels in stimulated cells. The blot was reprobed for total ERK levels as a control.

### 6.9: MEK inhibition in spreading WT BMMs produces a phenotype similar to spreading PAK1$^{-/-}$ BMMs

ERK activity has been implicated in cell spreading (Ishibe et al., 2004) and was shown to be decreased in PAK1$^{-/-}$ BMMs 10 minutes after adhesion (Figure 6.7). Using 1 $\mu$g/ml of the MEK inhibitor U0126, a role for ERK in BMM spreading was investigated.

Due to the reversible nature of the U0126 inhibitor, it was necessary to incubate the BMMs with the inhibitor whilst suspended in growth medium. The cells were then filmed spreading by timelapse microscopy (Figure 6.8 and Movies 6.3 and 6.4). The movies revealed that inhibition of MEK signalling in spreading WT cells produced a phenotype similar to that seen in PAK1$^{-/-}$ BMMs. MEK-inhibited BMMs produced less stable lamellipodia that appeared to frequently retract. This suggests that a possible reason why PAK1$^{-/-}$ BMMs have defects in spreading is due to a reduction in ERK signalling.

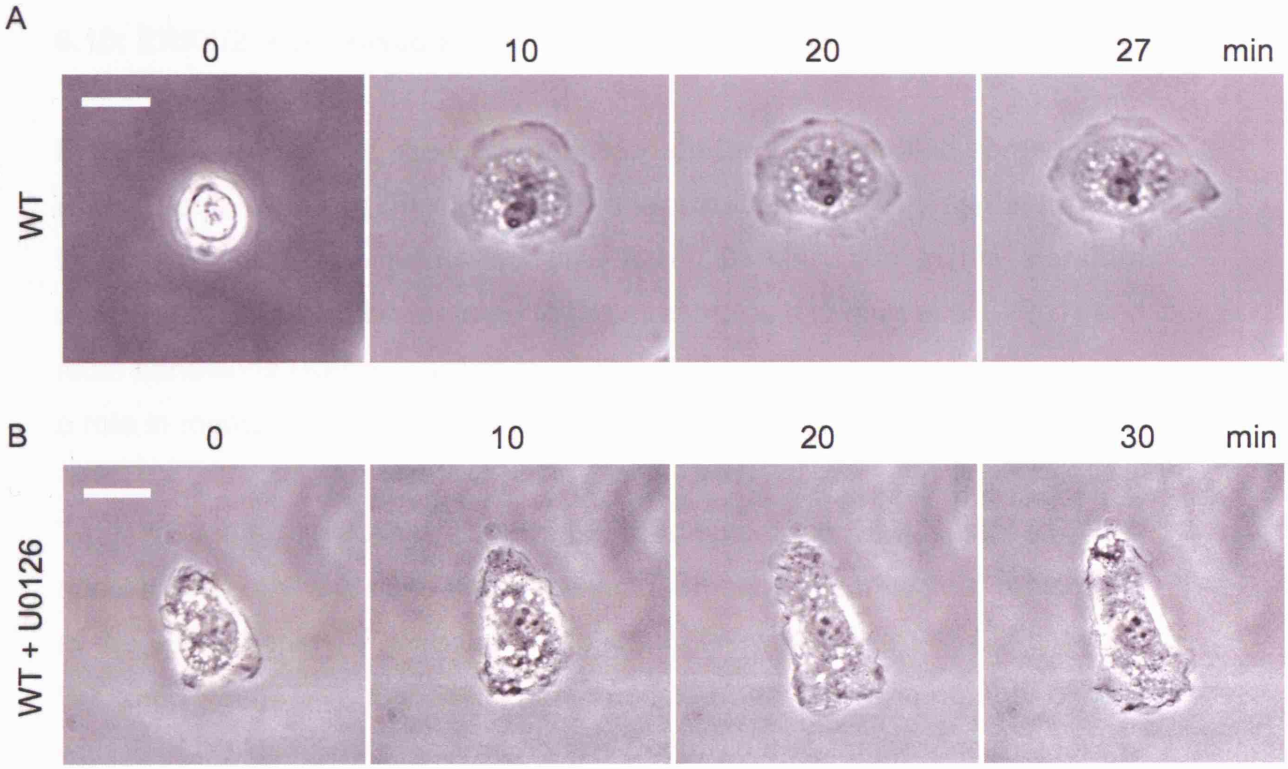



**Figure 6.8:** Inhibition of ERK with the U0126 MEK inhibitor leads to reduced spreading stability in WT BMMs

A) WT BMMs were suspended in growth medium for one hour and then replated onto a glass bottom microwell dish. Timelapse microscopy of spreading WT BMMs was performed using a x20 objective and 1 frame was acquired every 10 sec (Movie 6.3). The final image is after 27 min as the cell did not adhere until the 3rd minute of the movie. Images shown are cropped from a larger field of view for easier visualisation of spreading BMM. B) WT BMMs were suspended in growth medium for one hour containing 1 μg/ml U0126. BMMs were plated onto glass bottom microwell dishes still in the presence of the inhibitor. Timelapse microscopy was performed and 1 frame was acquired every 10 sec (Movie 6.4). Images are representative of at least 3 separate cells from 3 separate experiments, bars = 10 μm.

### 6.10: ERK1/2 is localised at the periphery of spreading BMMs

ERK1/2 signalling was shown to be important for the controlled spreading of BMMs upon adhesion (Figure 6.8). The localisation of ERK was determined to see if ERK1/2 is mislocalised in PAK1$^{-/-}$ BMMs. Data in the literature reports that ERK can be targeted to the membrane (Glading et al., 2001) and focal adhesions (Kermorgant et al., 2004), suggesting its signalling may play a role in membrane dynamics and spreading.

To investigate the localisation of ERK in spreading BMMs, an ERK1/2-specific antibody was used and observed ERK1/2 in a perinuclear region and at the cell periphery (Figure 6.9A). No clear differences were visible between WT and PAK1$^{-/-}$ BMMs and was confirmed by quantifying the number of cells with ERK1/2 localised at the membrane (Figure 6.9B), suggesting that loss of PAK1 is not inhibiting the correct localisation of ERK1/2 in BMMs.

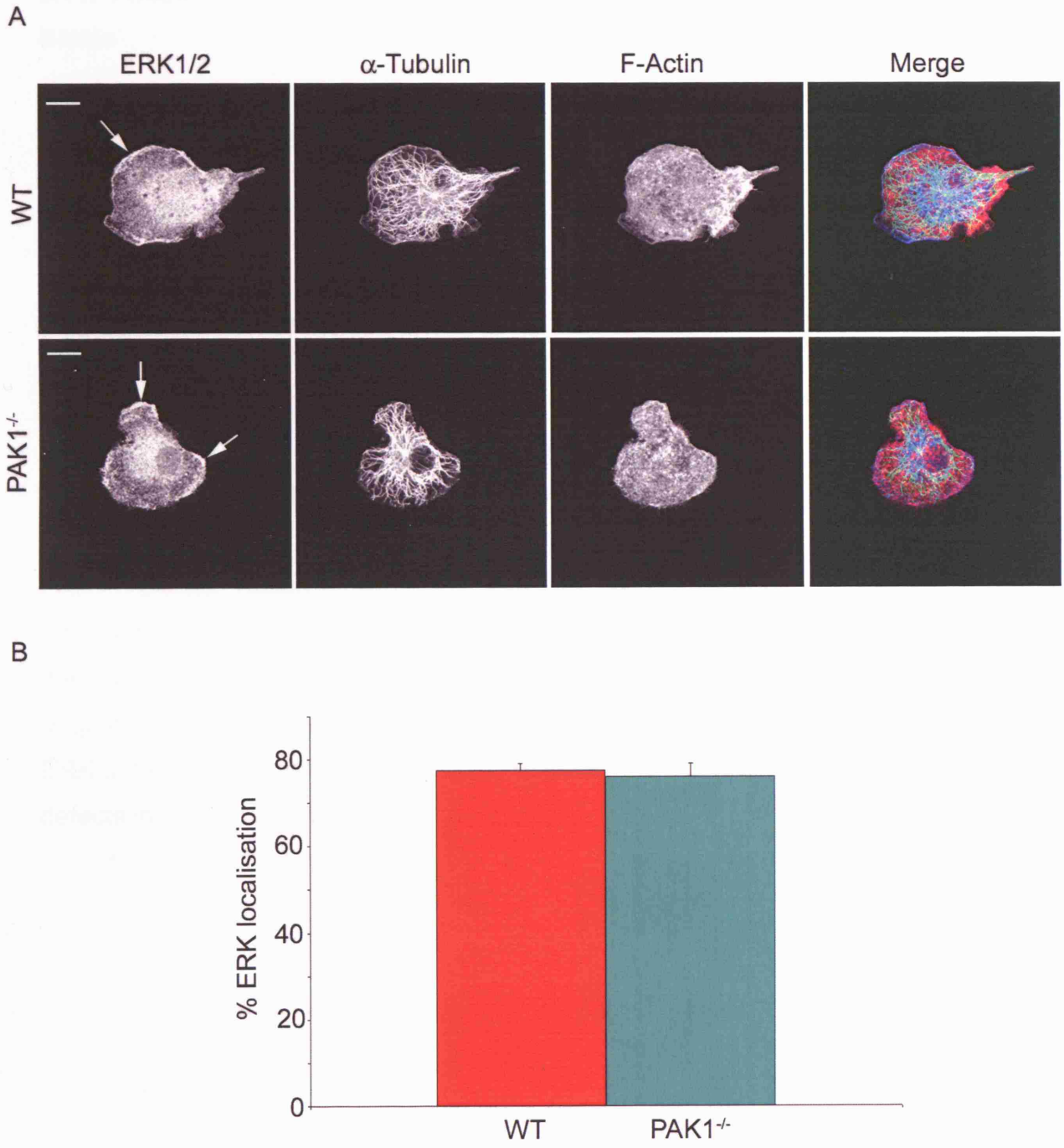



**Figure 6.9:** ERK1/2 localises to the cell periphery in spreading WT and PAK1$^{-/-}$ BMMs

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium and then plated onto glass coverslips in growth medium and allowed to adhere for 10 min before fixation. A) BMMs were stained for ERK1/2 (blue), α-tubulin (green) and F-actin (red) and images acquired by confocal microscopy. Images are representative of 2 separate experiments, bars = 10 μm, arrows indicate ERK localisation. B) Quantification of ERK1/2 at the plasma membrane. BMMs were scored for either having ERK1/2 localised at the membrane or not localised.Mean percentage +/- s.e.m of two separate experiments are shown. n = 60 (WT, 19 exp.1, 41 exp.2) n = 45 (PAK1$^{-/-}$, 19 exp.1, 27 exp.2).

## 6.11: Phosphorylated ERK is reduced at the cell membrane in PAK1$^{-/-}$ BMMs

A role for ERK in cell spreading has been suggested in the literature (Ishibe et al., 2004; Kermorgant et al., 2004) and by MEK inhibition of spreading WT BMMs (Figure 6.8). Although ERK localised to the plasma membrane in spreading WT and PAK1$^{-/-}$ BMMs (Figure 6.9), a possible role for PAK1 in phosphorylated ERK1/2 localisation was determined in WT and PAK1$^{-/-}$ BMMs.

WT and PAK1$^{-/-}$ BMMs were stained with the phosphorylation-specific ERK1/2 Thr202/Tyr204 antibody and confocal images were acquired. Phosphorylated ERK was present at the cell membrane in spreading WT and PAK1$^{-/-}$ BMMs. However, in PAK1$^{-/-}$ BMMs, fewer cells appeared to have localised phospho-ERK and showed a more diffuse distribution throughout the cell (Figure 6.10A). Quantification of phosphorylated ERK distribution (Figure 6.10B) confirmed that PAK1$^{-/-}$ BMMs have reduced phosphorylated ERK at the cell membrane, which could potentially be the cause of spreading defects in PAK1$^{-/-}$ BMMs.

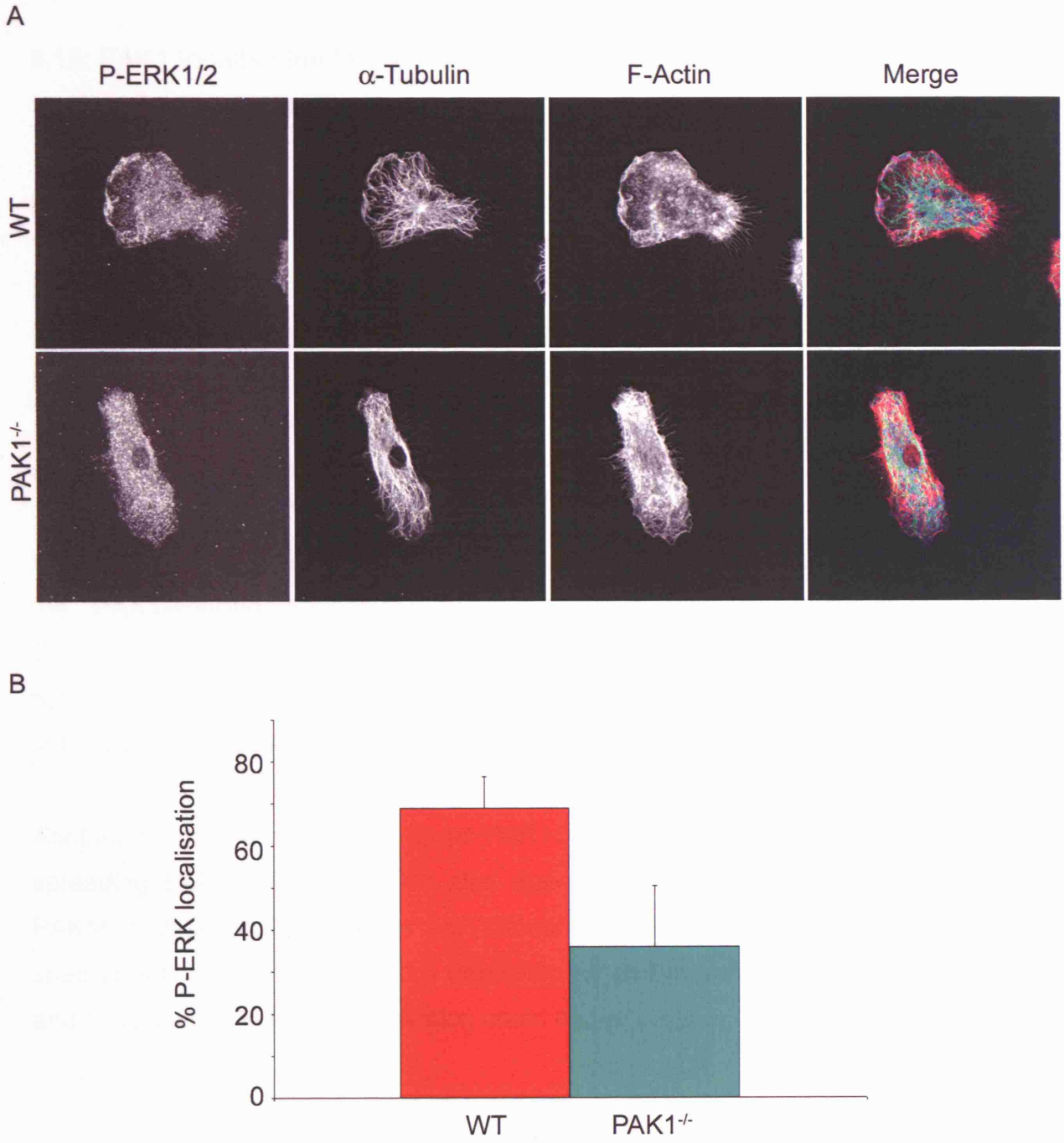



**Figure 6.10:** PAK1$^{-/-}$ BMMs have reduced phospho-ERK1/2 at the membrane during spreading.

WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips in growth medium and allowed to adhere for 10 min. A) BMMs were fixed and stained for P-ERK1/2 Thr202/ Tyr204 (blue), α-tubulin (green) and F-actin (red).Images were taken using a confocal microscope and are representative of 2 separate experiments. Bars = 10 μm, arrow indicates P-ERK localisation. B) Quantification of P-ERK at the cell membrane. BMMs were scored for either having P-ERK localised at the plasma membrane or not localised and the mean percentage of two independent experiments +/- s.d. are shown. n = 39 (WT, 21 exp.1,18 exp, 2) and n = 62 (PAK1$^{-/-}$, 48 exp. 1, 14 exp. 2).

## 6.12: PAK1 localisation in spreading BMMs could not be determined by immunofluorescence

Investigating the localisation of PAK1 in spreading cells is important for determining whether it gets targeted to the membrane like ERK. Inactive PAK1 is localised in the cytoplasm but moves to sites of cellular protrusion and focal adhesions upon activation by Rac and Cdc42 in fibroblasts and breast cancer cells (see review (Parrini et al., 2005)). FRET and FLIM microscopy also identified PAK localisation at the cell periphery (Del Pozo et al., 2002; Parsons et al., 2005).

WT and PAK1$^{-/-}$ BMMs spreading on coverslips were fixed and stained using the PAK1-specific antibody used on western blots (Cell Signalling Technology). Both cytoplasmic and some peripheral staining was observed, but this was also present in the PAK1$^{-/-}$ BMMs indicating that the PAK1 antibody is recognising other proteins (data not shown).

Another antibody that claims to target PAK1 (N-20, Santa Cruz) was used on spreading BMMs. This antibody also appeared to recognise proteins in PAK1$^{-/-}$ BMMs localised at the cell membrane (Figure 6.11). The PAK1-specific antibodies were therefore unreliable for use in immunofluorescence and conclusions on PAK1 localisation could not be made.

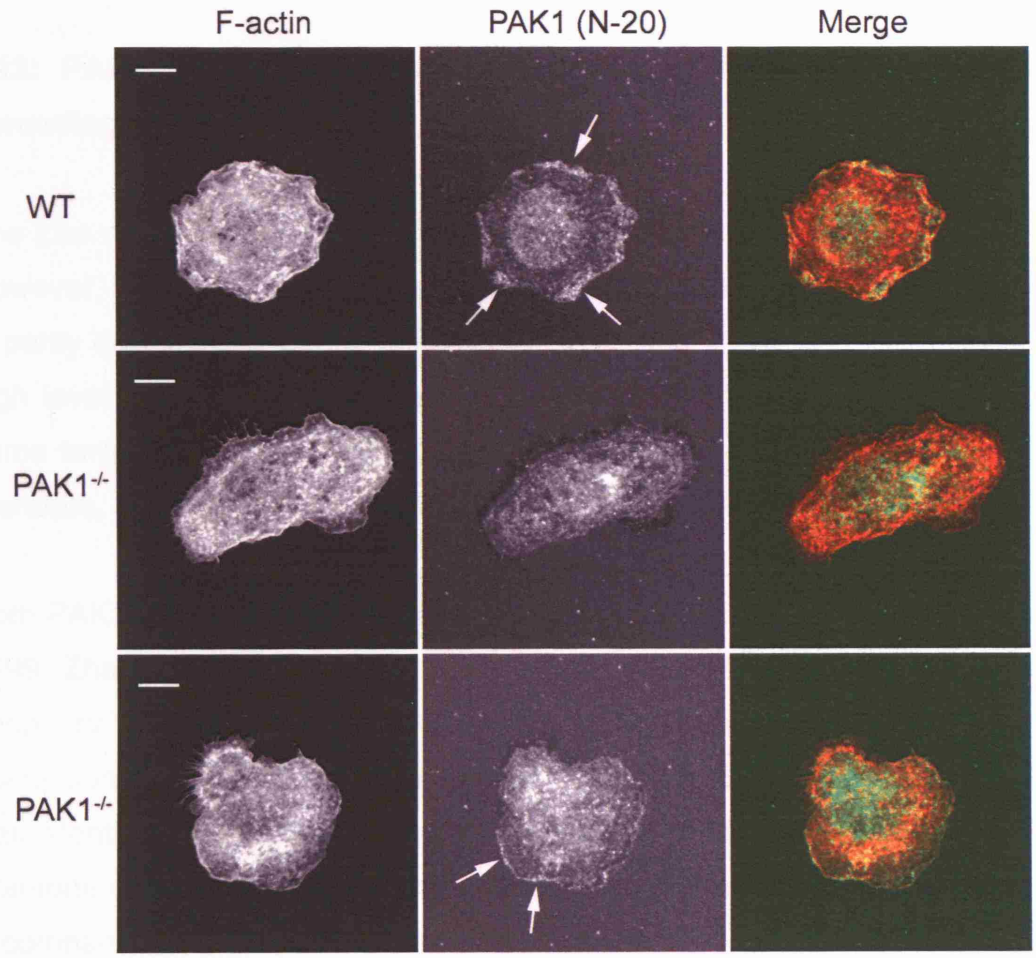



**Figure 6.11:** The PAK1 antibody is not specific in immunofluorescence

WT and PAK1$^{-/-}$ BMMs were plated on to glass coverslips in growth medium and allowed to adhere for 10 min. BMMs were fixed and stained using the N-20 anti-PAK1 antibody (green) and TRITC-phalloidin (red). Confocal images shown are representative of 2 independent experiments, arrows indicate localisation of PAK1 antibody, bars = 10 μm.

## 6.13: PAK2 and active Cdc42 co-localise at the cell periphery in spreading BMMs

The loss of PAK1 from BMMs results in impaired spreading upon adhesion. However, since PAK1$^{-/-}$ BMMs can spread and migrate, it is possible that this is partly due to redundancy within the PAK family. PAK1 and PAK2 have a high level of homology (Chapter 1.3) and have the potential to act on the same targets (see review (Bokoch, 2003)). The localisation of PAK2 was, therefore, investigated in spreading WT and PAK1$^{-/-}$ BMMs.

Both PAK1 and PAK2 are capable of being activated by Cdc42 (Gatti et al., 1999; Zhang et al., 1995) and active Cdc42 and PAK1 localise at the cell periphery promoting cytoskeletal reorganisation (Li et al., 2003). The interaction of active Cdc42 with another of its targets, N-WASp, however, was identified within endosomal compartments in breast carcinoma cells (Parsons et al., 2005) suggesting that GTPase signalling to different targets is compartmentalised.

Immunofluorescence was used to investigate PAK2 and active Cdc42 localisation in spreading BMMs. To visualise active Cdc42, a GST-N-WASp CRIB domain was utilised. An antibody specific for PAK2, whose specificity was established through PAK2 siRNA treatment of DU145 prostate cancer cells (personal communication, M. Bright), was used to visualise PAK2 localisation.

Confocal images showed that active Cdc42 localised at the cell periphery of spreading macrophages and co-localised with PAK2 (Figure 6.12). This suggests the possibility that upon adhesion, Cdc42 is targeted to the cell membrane and activates PAK2 to promote cytoskeletal reorganisation and potentially membrane extension. There were no changes between WT and PAK1$^{-/-}$ BMMs, suggesting PAK1 is not required for this localisation and that PAK2 either plays a separate role in cell spreading or can partially compensate for loss of PAK1.

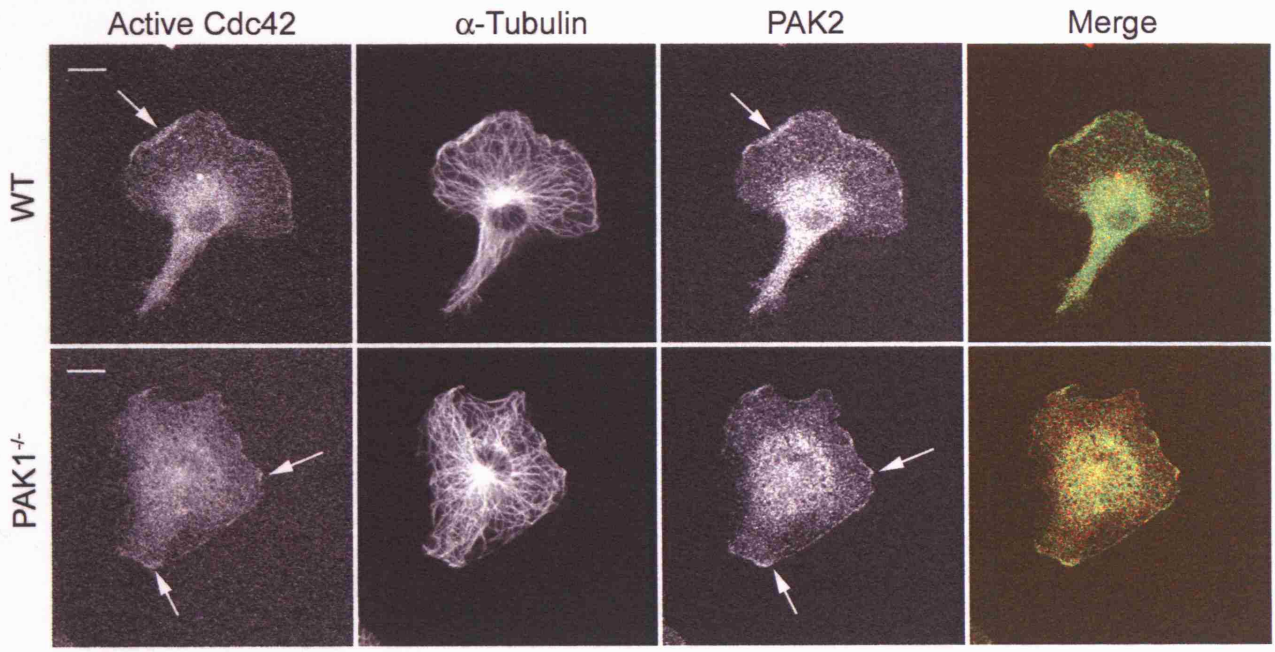



**Figure 6.12:** Active Cdc42 and PAK2 co-localise in WT and PAK1$^{-/-}$ BMMs

WT and PAK1$^{-/-}$ BMMs in growth medium were allowed to adhere to glass coverslips for 30 min and then fixed. BMMs were stained for active Cdc42 (red) using a GST-N-WASp CRIB domain, α-tubulin and PAK2 (green). Confocal images shown are representative of two separate experiments. Bar = 10 μm, arrows indicate sites of Cdc42 and PAK2 co-localisation.

### 6.14: BMMs express a number of β-PIX isoforms

An interaction between PAK1 and the PIX isoforms have been implicated in a number of processes including focal adhesion turnover and cell spreading (Chapter 1.3.5). Investigation of β-PIX expression and localisation was performed because of the identification of a β-PIX-PAK1 complex in fibroblast spreading (Ten Klooster et al., 2006).

Before determining whether β-PIX is localised at the cell periphery in spreading macrophages, it was first necessary to ensure that BMMs express β-PIX. Using cell lysates from a variety of cell types, WT and PAK1$^{-/-}$ BMMs were found to express the a/c, b, and d splice variants of β-PIX (Kim and Park, 2001). Due to the similarity in size of isoforms a and c, it was not possible to distinguish between them using western blotting (Figure 6.13). However, it did confirm that BMMs express a number of different β-PIX isoforms. Isoform expression was cell type specific: NIH3T3 and PC3 cells expressed the a/c and d isoforms whilst HT29 and DU145 cells expressed only the a/c β-PIX isoforms. All the macrophage cell lines (Raw 264.7, J774 and IC21) expressed the same β-PIX isoforms as the primary macrophages.

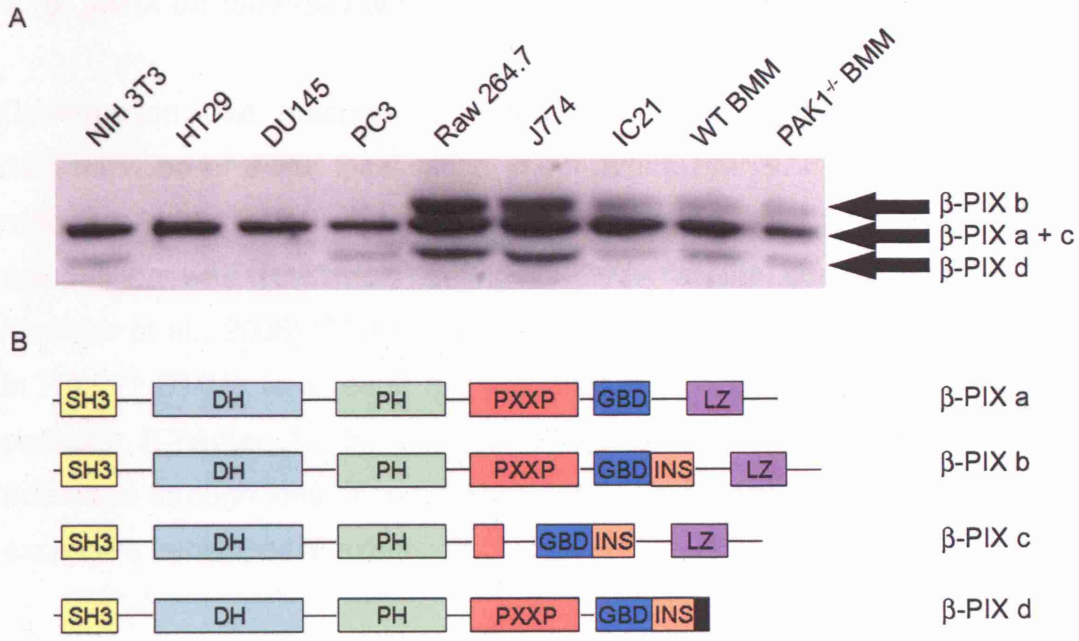



**Figure 6.13:** *β*-PIX isoform expression in primary macrophages and various cell lines.

A) Cell lysates from various cell types were separated by SDS-PAGE and analysed by western blotting with a *β*-PIX-specific antibody. Cells: NIH-3T3 = mouse fibroblasts, HT29 = human colon cancer, DU145 = human prostate cancer brain metastasis, PC3 = human prostate cancer, Raw 264.7, J774, IC21 = mouse macrophages. Results shown are representative of two separate experiments. B) Schematic representing the 4 *β*-PIX splice-variant isoforms identified. Domain abbreviations: SH3 = Src homology domain 3, DH = Dbl homology domain, PH = Pleckstrin homology domain, PXXP = Proline-rich region, GBD = GIT-binding domain, INS = Insert region, LZ = Leucine zipper domain, Black box = Novel 11 amino acid insert. Adapted from (Kim and Park, 2001).

### 6.15: β-PIX co-localises with active Cdc42 at the cell periphery

Confirmation that macrophages expressed β-PIX (Figure 6.13) allowed determination of β-PIX localisation in spreading BMMs. It has recently been reported that PAK1 regulates cell spreading in fibroblasts through competition with Rac1 for binding to β-PIX at the cell membrane (Ten Klooster et al., 2006). This raises the possibility that the defect in spreading in PAK1$^{-/-}$ BMMs is a result of deregulation of a Cdc42-β-PIX-PAK1-Rac1 complex (Chapter 1.3.5). Loss of PAK1 could result in aberrant Rac1 activation through loss of competition for β-PIX binding which could result in excessive lamellipodial extension.

To test this model, the localisation of active Cdc42 and β-PIX was investigated using immunofluorescence. Cells were stained using an anti-β-PIX antibody and the GST-N-WASp CRIB domain. In both WT and PAK1$^{-/-}$ BMMs, active Cdc42 and β-PIX co-localised at the leading edge after 30 min (Figure 6.14A) and 15 min of adhesion (Figure 6.14B). The cells at 30 min are generally more polarised than at earlier time points and make identification of co-localisation between Cdc42 and β-PIX easier. However, repeating the staining at an earlier time point (15 minutes) also showed co-localisation of the proteins.

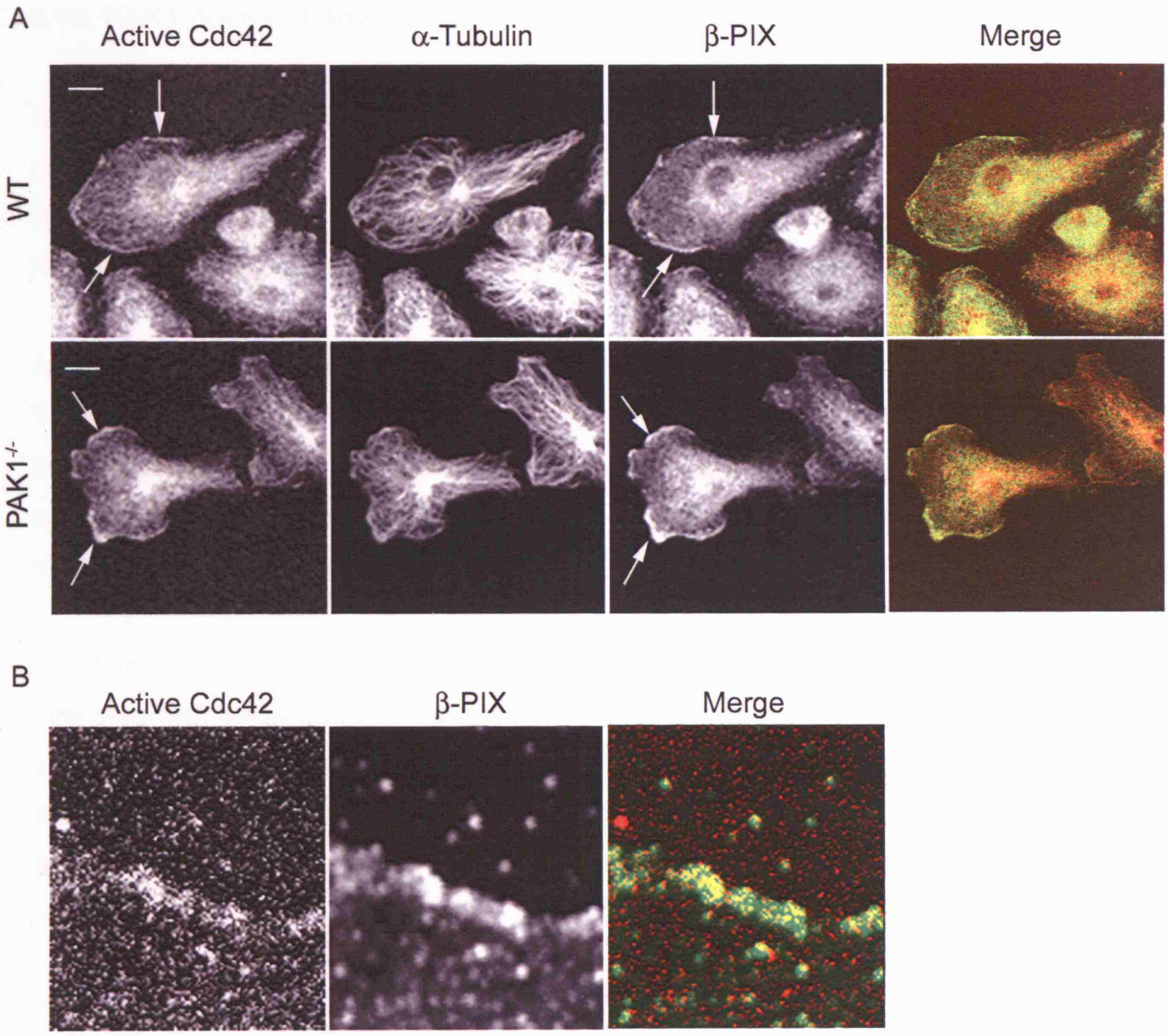



**Figure 6.14:** Active Cdc42 colocalises with β-PIX at cell edge in both WT and PAK1$^{-/-}$ BMMs.

WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips in growth medium and allowed to adhere for 30 min before fixation. A) Fixed cells were stained for active Cdc42 (red) with a GST-N-WASp CRIB domain, α-tubulin and β-PIX (green). Images were acquired using a confocal microscope. B) WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips in growth medium and fixed 15 min after adhesion. Cells were stained for active Cdc42 (red) and β-PIX (green) and images were acquired using a confocal microscope. Images are representative of two separate experiments, bars = 10 μm.

### 6.16: PAK1 does not influence Rac1 and β-PIX co-localisation

Active Cdc42 and β-PIX and active Cdc42 and PAK2 co-localised at the cell periphery in spreading macrophages (Figure 6.12 and 6.14). To investigate whether Rac1 is also present at the cell periphery in spreading BMMs, a Rac1 antibody was used.

As a control for Rac1 staining, $Rac1^{-/-}$ BMMs were used (Wells et al., 2004). This indicated that the Rac1 antibody was not entirely specific in immunofluorescence (Figure 6.15B). However, staining was much reduced at the cell periphery compared to WT BMMs and allowed the assumption that strong staining at the membrane was indicative of Rac1 localisation.

Co-staining of WT and $PAK1^{-/-}$ BMMs with the Rac1 and β-PIX antibodies indicated that these proteins localise in similar areas of the cell membrane in spreading cells regardless of PAK1 expression (Figure 6.15A). This suggests that Rac1 and β-PIX are both targeted to the membrane during spreading and that PAK1 is not required for this localisation.

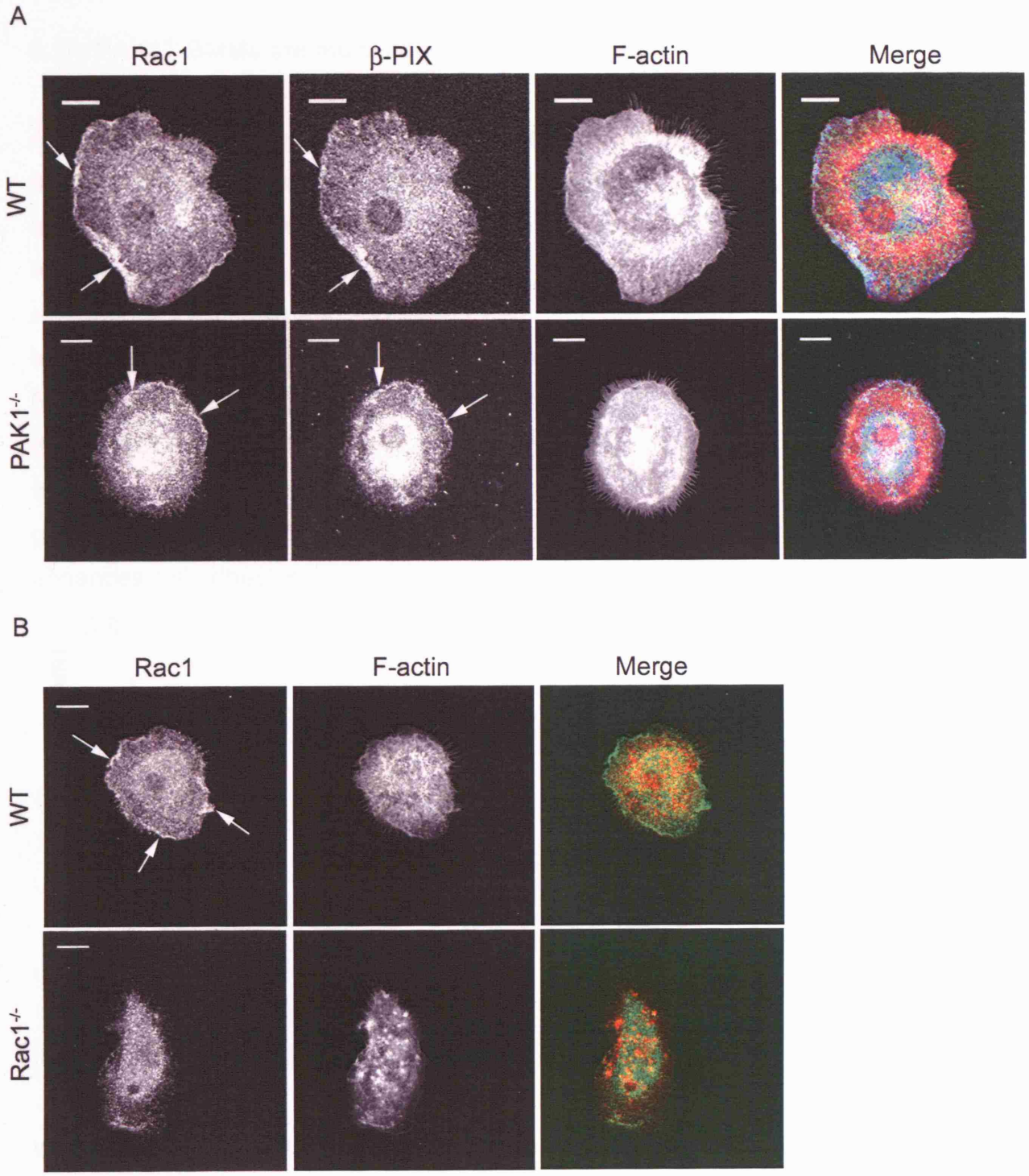



**Figure 6.15:** Rac1 co-localises with β-PIX in the absence of PAK1

WT, PAK1$^{-/-}$ and Rac1$^{-/-}$ BMMs were plated onto glass coverslips in growth medium and allowed to adhere for 10 min. BMMs were fixed and stained with (A) an anti-Rac1 antibody (blue), an anti-β-PIX antibody (green) and with TRITC-phalloidin to localise F-actin (red) or (B) with an anti-Rac1 antibody (green) and with TRITC-phalloidin (red). The same contrast is used to show the reduction of Rac1 staining in Rac1$^{-/-}$ BMMs. Representative confocal images are shown, similar results were observed in 2 separate experiments. Bars = 10 μm.

### 6.17: PAK1$^{-/-}$ BMMs are more adhesive than WT BMMs

As well as defects in cell signalling (Figure 6.6), it was possible that an altered ability to adhere could contribute to the different spreading behaviour of PAK1$^{-/-}$ BMMs. PAK has been implicated in a number of pathways that regulate cell adhesion (Chapter 1.3.5). To investigate whether loss of PAK1 affected BMM adhesion, a MTT assay was performed (Chapter 2.5.15). WT and PAK1$^{-/-}$ BMMs were plated onto tissue culture plastic for between 5 and 60 minutes and the number of adherent cells was quantified by the optical density (OD) of the BMM medium after MTT addition and cell lysis. This indicated that PAK1$^{-/-}$ BMMs adhere to tissue culture plastic at a significantly greater rate than WT BMMs (Figure 6.16) suggesting that loss of PAK1 enhances cell adhesion.

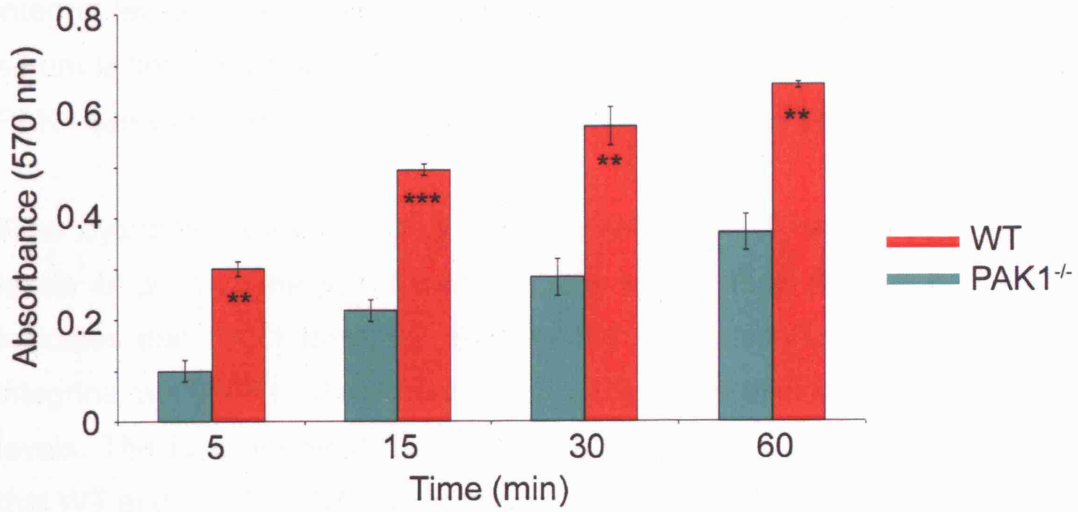



**Figure 6.16:** PAK1$^{-/-}$ BMMs adhere more rapidly than WT BMMs.

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium. BMMs were suspended in growth medium and allowed to adhere to tissue culture plastic for between 5 and 60 min. BMMs were washed in PBS and grown for a further 2 hours in growth medium containing 25 mM MTT. BMMs were lysed with 5% SDS for 1 hour and the optical density (OD) of the lysates was measured at 570 nm. Results are the mean +/- s.e.m of three separate experiments, Student's t-test analysis showed a significant difference between WT and PAK1$^{-/-}$ BMM adhesion (** = $p < 0.01$, *** = $p < 0.001$).

## 6.18: PAK1$^{-/-}$ BMMs show no changes in $\beta_1$ or $\beta_2$ integrin expression levels

It is possible that the increased adhesion of PAK1$^{-/-}$ BMMs is due to a decrease in adhesion turnover. However, no obvious defects in $\beta$-PIX localisation, the protein that targets PAK to integrin-based adhesions, were observed in PAK1$^{-/-}$ cells (Turner et al., 1999 and Figure 6.15).

Another possibility is a change in the levels of adhesion proteins expressed on the cell surface. PAK1 has been implicated in the regulation of transcription via the MAPK pathway (Figure 4.2) and the NF-$\kappa$B transcription factor (Dadke et al., 2003) making it possible that PAK1 plays a role in the regulation of integrin expression. To investigate this possibility, $\beta_1$ and $\beta_2$ integrin levels were studied because one of the principle matrix proteins in serum is fibronectin, a binding target of $\beta_1$ integrin (Herard et al., 1996), and PAK1 can signal to the $\alpha_M\beta_2$ integrin heterodimer (Jones et al., 1998).

Flow cytometry showed that WT and PAK1$^{-/-}$ BMMs expressed identical levels of $\beta_1$ and the $\beta_2$ integrins on the cell surface (Figure 6.17). This indicates that PAK1 does not regulate the expression levels of $\beta_1$ and $\beta_2$ integrins, and PAK1$^{-/-}$ BMMs are not more adhesive due to elevated integrin levels. The flow cytometry also indicates, via the forward and side scatter, that WT and PAK1$^{-/-}$ BMMs were of a similar size.

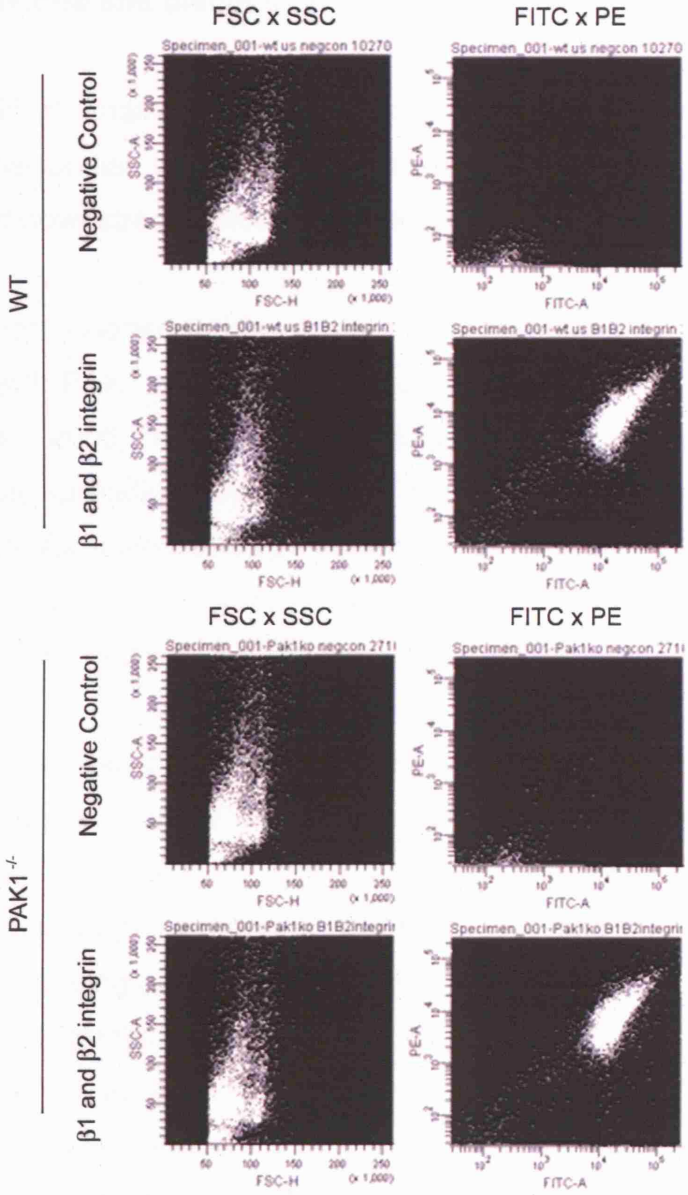



**Figure 6.17:** WT and PAK1$^{-/-}$ BMMs express equal levels of $\beta$1 and $\beta$2 integrins

WT and PAK1$^{-/-}$ BMMs were cultured in growth medium. BMMs were suspended in FACS buffer and stained with fluorescently conjugated $\beta$1 (FITC) and $\beta$2 (PE) integrin antibodies. FACS analysis was used to determine the integrin expression on the cell surface of WT and PAK1$^{-/-}$ BMMs. Unstained BMMs were used as negative controls; results are representative of 2 separate experiments.

## 6.19: Conclusions and discussion

Rac1 is a critical small GTPase involved in spreading through its ability to promote lamellipodial extension (Wells et al., 2004) via a number of upstream and downstream effectors (Chapter 1.3.5).

PAK1 has been reported to play a role in cell spreading. Competition for β-PIX binding with PAK1 regulates Rac1 activation at the cell membrane (Ten Klooster et al., 2006), and PAK1 was also activated downstream of Rac1 during platelet spreading (Suzuki-Inoue et al., 2001). Loss of VASP also increased cell spreading through enhanced activation of Rac1 and PAK1 (Garcia Arguinzonis et al., 2002) whilst the spreading inhibitor, Ephrin A1, acts through Rac1 and PAK1 (Deroanne et al., 2003).

$PAK1^{-/-}$ BMMs spread and elongate during adhesion at a greater rate than WT BMMs suggesting that PAK1 is involved in the regulation of membrane extension upon adhesion. In random migration assays (Figure 5.2), no obvious increase in cell area was observed with time suggesting that the enhanced spreading in adhering $PAK1^{-/-}$ BMMs was a temporary consequence of deregulated membrane extension. $PAK1^{-/-}$ BMMs retracted back to a spread area similar to that of WT BMMs and the extent of membrane retraction undergone by $PAK1^{-/-}$ BMMs was suggested by their spiky appearance. After $PAK1^{-/-}$ BMMs adhere, they may spread to too great an area and subsequently require greater membrane retraction. This indicates that $PAK1^{-/-}$ BMMs are capable of membrane retraction. It is unclear whether the ability of $PAK1^{-/-}$ BMMs to return to a steady-state area is due to membrane retraction or contraction. Membrane retraction involves the internalisation of plasma membrane resulting in a net reduction of cell size, whereas contraction involves an active process of pulling the plasma membrane in to reduce cell area. PAK1 regulates contraction through inhibition of MLCK (Sanders et al., 1999; Wirth et al., 2003) and MLCP (Takizawa et al., 2002). MLCK inhibition in mouse embryonic fibroblasts inhibited spreading (Giannone et al., 2004) indicating a role for MLCK in spreading. Attempts to observe phospho-MLC levels in WT and $PAK1^{-/-}$

BMMs cell lysates after adhesion were unsuccessful presumably due to low levels (data not shown). It is possible though, that only low levels of phosphorylation are required to produce a change in cell contractility. PAK1 is also required for contraction of a 3D collagen matrix by human fibroblasts downstream of PDGF and LPA signalling (Rhee and Grinnell, 2006). However, it appears that in BMMs, the loss of PAK1 does not inhibit cell contraction although defective MLCK activity could participate in the observed spreading phenotype.

During adhesion, WT BMMs produce stable lamellipodia that gradually encompass more of the cell perimeter. PAK1$^{-/-}$ BMMs also produce lamellipodial extensions upon adhesion but they are more numerous, smaller, less stable and frequently retract back into the cell body. As the movies produced were phase-contrast, the contrast was insufficient for automated spread area analysis, but analysis with MetaMorph software suggested PAK1 is not critical for lamellipodial extension but is required for lamellipodial persistence. However, the PAK1$^{-/-}$ BMMs spread quicker than WT BMMs although the lamellipodia produced are less stable and retract more frequently. It is possible that the PAK1$^{-/-}$ BMMs exhibit a greater spread area in the confocal studies because of the increased number of lamellipodia that are produced around the cell. The production of multiple unstable lamellipodia may also enhance polarisation explaining why PAK1$^{-/-}$ BMMs were more elongated during spreading. This polarisation could also affect spread area through production of a migratory phenotype.

To study the PAK1$^{-/-}$ BMM spreading defect in more detail, total internal reflection fluorescence (TIRF) microscopy could be utilised. This would allow visualisation of the basal 100 nm of the cell and observation of the macrophage membrane as it extended and retracted after adhesion (Giannone et al., 2004). This would indicate the speed of spreading and the dynamics of membrane extension.

The reduction in lamellipodial stability observed in PAK1$^{-/-}$ BMMs is possibly also responsible for other phenotypes observed, i.e. the increased number

and reduced size of lamellipodia. One possible explanation is that PAK1 plays a role in localising lamellipodial extension. Without PAK1, lamellipodia may simultaneously extend around the cell, exhausting the pool of effector proteins and resulting in increased lamellipodial collapse. PAK1 was required for regulating Rac1 activity at the cell membrane through binding to β-PIX (Ten Klooster et al., 2006). Loss of PAK1 may allow excessive Rac1 binding to β-PIX leading to Rac1 activation and enhanced membrane protrusion. A key protein involved in lamellipodial stability that may become exhausted is cofilin (Ghosh et al., 2004), a downstream target of PAK1 which is inhibited via LIMK (Edwards et al., 1999).

PAK1-dependent signalling pathways that inhibit lamellipodial collapse may also be absent in $PAK1^{-/-}$ BMMs. Lamellipodial collapse is frequently studied in neuronal growth cones due to the ability of extracellular cues to induce collapse. For example, Ephrin-A5 promoted retinal growth cone collapse through activation of ROCK (Wahl et al., 2000) in conjunction with Abl family kinases (Harbott and Nobes, 2005). CA Rac1 and RhoA abolished CNS myelin-induced growth cone collapse whilst DN Rac1 and Cdc42 inhibited growth cone collapse in response to collapsin-1 (Kuhn et al., 1999). It is possible, therefore, that loss of PAK1 downstream of Rac1 could result in lamellipodial instability.

ERK is involved in regulating spreading (Chapter 1.3.6). $PAK1^{-/-}$ BMMs had reduced phosphorylated ERK1/2 after adhesion although no changes in MEK phosphorylation were observed. This suggests that adhesion induces PAK1 stimulation of ERK in a MEK-independent manner. Formation of a MEK1-ERK complex was shown to be PAK1-dependent and required the phosphorylation of MEK1 at Ser298 (Eblen et al., 2004), a site believed to be a PAK1-specific target although this was not observed in BMMs (Chapter 4.4). Other PAK1 phosphorylation sites may exist, however, and it could be the absence of these that reduces ERK activation upon adhesion. Co-immunoprecipitation of a MEK1-ERK complex from adhering BMMs, however, was not possible (data not shown). PAK1 may act as a scaffold protein at adhesions bringing Raf-MEK1-ERK into a complex to promote

activation of ERK upon adhesion (Sundberg-Smith et al., 2005). Loss of PAK1 may prevent complex formation and cause a reduction in ERK activity and resultantly, lamellipodial stability. p38 also promoted cell adhesion downstream of Rac (Schindeler et al., 2005) but no changes in phosphorylated p38 levels were observed in adhering $PAK1^{-/-}$ BMMs.

Only ERK1/2 had a clear phosphorylation defect upon $PAK1^{-/-}$ BMM adhesion. It is not possible to rule out an involvement for the other proteins studied as PAK may act as a scaffolding protein, bringing these proteins into a complex. It could be the loss of protein complexes which results in reduced activation of signalling pathways not identified here. To investigate ERK's role in lamellipodial stability, MEK was inhibited as no specific ERK inhibitors are currently available. They are, however, being developed as potential anti-cancer therapies (Hancock et al., 2005). MEK-inhibited WT BMMs also produced numerous unstable lamellipodia. This indicated that ERK activity is required for the stable production of lamellipodia. However, as already stated, the mechanism by which PAK regulates ERK activity is still unclear. ERK1/2 localised to the cell periphery in spreading BMMs regardless of PAK1 expression. ERK was previously shown to bind and phosphorylate paxillin at the cell periphery (Ishibe et al., 2004) possibly through FAK phosphorylation of paxillin allowing Crk and resultantly, ERK targeting and activation (Petit et al., 2000; Subauste et al., 2004). However, PAK may also act as a scaffold protein for ERK, Raf and MEK at focal complexes (Sundberg-Smith et al., 2005). This appears unlikely in BMMs as the loss of PAK1 should disrupt the localisation of ERK, however, PAK2 may be able to replace PAK1.

Phosphorylated ERK1/2 was concentrated at the cell periphery in spreading WT BMMs but was reduced in $PAK1^{-/-}$ BMMs implicating PAK1 in ERK activation at the leading edge. Inhibition of ERK in CSF-1-stimulated spreading macrophages led to hyper-phosphorylated FAK (Rovida et al., 2005). This could be significant for the regulation of spreading because FAK regulates focal contact assembly and disassembly, cytoskeletal stability and activation of Rho GTPases (see reviews (McLean et al., 2005; Mitra et al.,

2005)). In haematopoietic cells the FAK-related kinase Pyk2 is also expressed. Pyk2 is similar to FAK (65% amino acid similarity) but has differences in phosphorylation sites and downstream targets (see review (Duong and Rodan, 2000)). However, Pyk2 also targets the cytoskeleton via activation of the Rho GTPases, relays integrin signalling and activates the MAPK pathways (see review (Avraham et al., 2000) and (Sekimoto et al., 2005)). At present, there is no evidence that ERK regulates Pyk2 activity, however, given the similarity in sequence and function between FAK and Pyk2, ERK regulation of Pyk2 is possible. The Pyk2 phosphorylation site, Tyr402, is involved in the binding of SH2 domains (Lakkakorpi et al., 2003) and integrin $\beta_3$ (Butler and Blystone, 2005). Autophosphorylation of Tyr402 was essential for spreading in osteoclasts, (Lakkakorpi et al., 2003) and the formation of podosomes in macrophages. Anti-sense treatment of Pyk2 inhibited spreading and migration (see review (Duong and Rodan, 2000)) and $Pyk2^{-/-}$ macrophages have defects in membrane ruffling and lamellipodial contractility (Okigaki et al., 2003). However, PAK1 did not appear to regulate Pyk2 Tyr402 in BMMs. However, other phosphorylation sites not studied in Lakkakorpi *et al.* may be PAK1-dependent and affect spreading.

Another link between ERK and spreading involves regulation of cytoskeletal proteins such as vinexin and cortactin (see review (Yoon and Seger, 2006)). Vinexin is a binding partner of vinculin and is localised at focal adhesions and sites of cell: cell contact. ERK2 binds and phosphorylates vinexin $\beta$ upon adhesion and growth factor stimulation and potentially affects cell spreading (Mitsushima et al., 2004). Cortactin is a PAK1 target that is involved in formation of lamellipodia and membrane ruffling (Weed et al., 1998) and is essential for integrin-mediated spreading (Ilies et al., 2006b). ERK also phosphorylates cortactin allowing activation of WASP and N-WASP and resultantly, stimulation of the Arp2/3 complex (Martinez-Quiles et al., 2004), although cortactin may not require phosphorylation to induce actin polymerisation (Ilies et al., 2006a). It was not possible to localise PAK1 in BMMs by immunofluorescence. However, Cdc42 and PAK2 co-localise at the cell periphery and within areas of membrane extension in WT and $PAK1^{-}$

$^{-/-}$ BMMs. PAK2 may therefore have a separate role in spreading alongside PAK1 rather than replacing it although PAK2 cannot completely compensate for PAK1 deletion during spreading. Use of RNAi or expression of the PAK AID would be useful to determine the role of PAK2 in spreading.

β-PIX was implicated in the activation of Rac to promote spreading (Ten Klooster et al., 2006) and is involved in signalling to the MAPK pathways (Lee et al., 2001), in adhesion formation (Rosenberger and Kutsche, 2005; Webb et al., 2005) and disassembly (Zhao et al., 2000). β-PIX localisation, its adaptor function and its GEF activity are, therefore, likely to have a key function in cell spreading. β-PIX was also implicated in neurite outgrowth downstream of ERK-PAK2 signalling (Shin et al., 2002). WT and PAK1$^{-/-}$ BMMs and other macrophage cell lines express the same β-PIX isoforms. PAK1, however, was not required for the localisation of β-PIX or Cdc42 in spreading BMMs suggesting that mislocalised activity from β-PIX or Cdc42 is unlikely to be the cause of reduced lamellipodial stability. Ten Klooster *et al.* postulated that Cdc42 activation of PAK1 at the leading edge is required for its dissociation from β-PIX allowing Rac1 binding (Ten Klooster et al., 2006). Rac1 staining co-localised with β-PIX at the cell periphery in spreading WT and PAK1$^{-/-}$ BMMs indicating that PAK1 is not required. With the loss of PAK1, an increase in Rac1 binding and activity may be expected at the cell periphery leading to excessive spreading. A similar model suggests Src and FAK promote an Nck-PAK-PIX-GIT2 complex to bind paxillin which reduces Rac activity via GIT2 inactivation of Arf6 (Frank et al., 2006; Nishiya et al., 2005). Reduced ERK activation of FAK may also disrupt localisation of this complex enhancing cell spreading (Brown et al., 2005) (Figure 6.18). Co-immunoprecipitation experiments of Rac1 and β-PIX would determine any changes in Rac1-β-PIX binding. It is also possible that despite no apparent changes in Rac1 levels at the cell periphery, there could be an increase in Rac1 activity in PAK1$^{-/-}$ BMMs as observed in PAK1$^{-/-}$ fibroblasts (Ten Klooster et al., 2006). Rac1 pull-down assays using a GST-PAK CRIB domain could be performed to determine active Rac1 levels in BMMs (Wojciak-Stothard and Ridley, 2003).

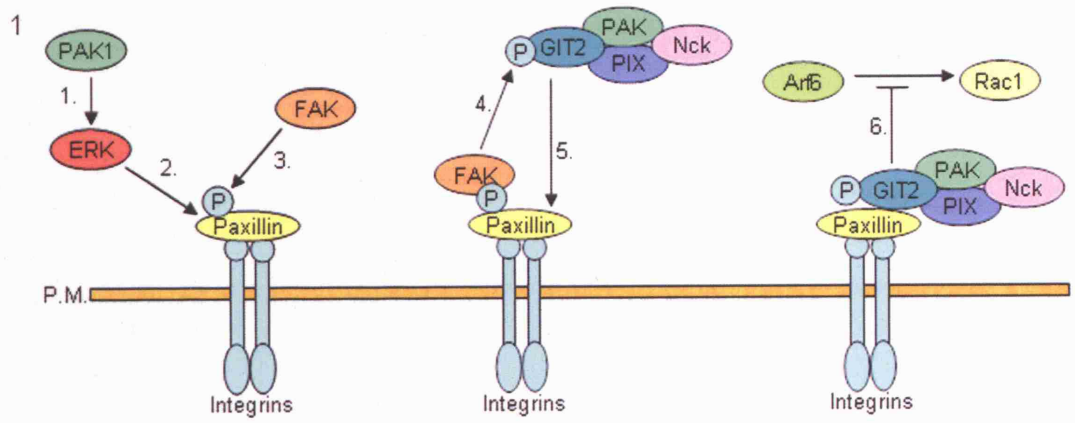
As well as regulating signalling pathways affecting the cytoskeleton, it is possible that loss of PAK1 affects BMMs adhesion. The increased rate of lamellipodial collapse and retraction could be due to a decrease in focal contact adhesion formation within the growing lamellipodium. Another family of proteins that act as Rac effectors, the WAVE proteins, have been implicated in lamellipodia stabilisation through the formation of adhesions (Yamazaki et al., 2005). PAK1 may also be involved in this process downstream of Rac, since PAK1$^{-/-}$ BMMs adhered at a significantly greater rate than WT BMMs despite expressing the same levels of $\beta_1$ and $\beta_2$ integrins.

A further possibility is that PAK1$^{-/-}$ BMMs do not have an increased ability to adhere compared to WT BMMs but have an increased ability to process MTT. A mitochondrial dehydrogenase cleaves the tetrazolium ring of MTT in viable cells producing purple formazan crystals which accumulate within the cell. No evidence suggests PAK1 regulates a mitochondrial dehydrogenase, although it does function at the mitochondria (Jin et al., 2005). However, an increase in PAK1$^{-/-}$ BMM metabolism could also influence MTT cleavage producing false positive results.

If PAK does regulate adhesion, it could do so through a number of signalling pathways including FAK, GIT and ERK (Brown et al., 2005; Eblen et al., 2002; Zhao et al., 2000). A potential link between ERK signalling and adhesion is through its ability to phosphorylate paxillin at Ser83, enhancing cell adhesion and spreading (Ishibe et al., 2004; Liu et al., 2002) through promotion of FAK binding to paxillin (Ishibe et al., 2004). This is supported by data implicating ERK in the regulation of FAK activity in CSF-1 stimulated macrophages (Rovida et al., 2005). Another possible explanation is that the loss of PAK1 affects the turnover of adhesions. PIX, through its ability to bind PAK1 and GIT1, promotes the disassembly of focal adhesions (Zhao et al., 2000) whilst GIT2 inhibits Cdc42-induced focal adhesion turnover (Frank et al., 2006). However, macrophages do not form focal adhesions but form smaller focal contacts (Allen et al., 1997) although these may also be regulated by PAK1, PIX and GIT. Podosome formation within smooth muscle

cells is also a PAK1-β-PIX dependent process (Webb et al., 2005). A defect in podosome formation could affect macrophage adhesion. Cortactin localises in podosomes and its phosphorylation helps regulate podosome stability and turnover (Zhou et al., 2006). Cortactin is a PAK1 (Vidal et al., 2002; Weed et al., 1998) and an ERK target (Martinez-Quiles et al., 2004). It is possible that downstream of PAK1, ERK phosphorylation of cortactin promotes podosome formation via WASp and N-WASp. To investigate this further, immunofluorescence staining of actin, cortactin, paxillin and Pyk2 should show adhesions within WT and PAK1$^{-/-}$ BMMs.

To summarise, PAK1 regulates the controlled spreading of macrophages upon adhesion. PAK1$^{-/-}$ BMMs temporarily spread to a greater area than WT BMMs but do so with reduced lamellipodial persistence and stability. Despite the reduction in lamellipodial stability, β-PIX, ERK1/2, PAK2, Rac1 and active Cdc42 are localised normally at the cell periphery, although P-ERK1/2 is reduced at the periphery consistent with a role in spreading. Reduced lamellipodial stability is not linked to reduced adhesion since PAK1$^{-/-}$ BMMs adhered more quickly than WT BMMs.



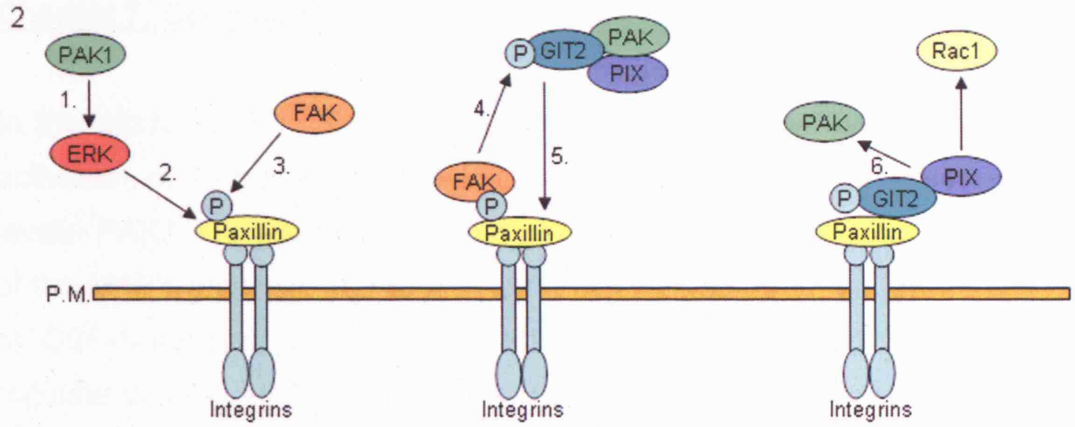



**Figure 6.18:** Possible mechanisms regulating BMM spreading by PAK1.

1) Upon adhesion, PAK1 is activated and promotes the activation of ERK. ERK is localised to the cell periphery and phosphorylates paxillin, promoting FAK binding and activation. FAK phosphorylation of GIT2 promotes targeting of a GIT2-$\beta$-PIX-PAK1-Nck complex to the adhesion and inhibition of Arf6 activity towards Rac. 2) Similar to 1) except FAK phosphorylation of GIT2 promotes localisation of a GIT2-$\beta$-PIX-PAK1 complex. Cdc42 activation of PAK1 promotes release from $\beta$-PIX, allowing Rac1 binding and activation. P.M = plasma membrane.

# Chapter 7: Concluding remarks and future perspectives

In this study, CSF-1 stimulation of BMMs was shown to induce a transient activation of PAK1 whilst also inducing a later increase in PAK1 protein levels. PAK1 activation downstream of CSF-1 promoted the phosphorylation of the MAPK pathways, LIMK and Op18 but was not required for cell motility or CSF-1-induced chemotaxis. PAK1, possibly via ERK1/2, however, did regulate lamellipodial stability in BMMs during adhesion-induced spreading. PAK1, therefore, was required for CSF-1-induced signalling and adhesion-induced spreading but was not critical for macrophage polarisation, migration or chemotaxis.

## 7.1: PAK1 and macrophage function

Circulating monocyte extravasation into the tissues is critical for immune responses and requires both adhesion and spreading upon the endothelium (Barreiro et al., 2004). $PAK1^{-/-}$ macrophages had reduced lamellipodial extension and stability during spreading (Chapter 6. 4) but also enhanced cell adhesion (Chapter 6.16). The changes in $PAK1^{-/-}$ BMMs, therefore, could indicate a role for PAK1 in extravasation. To investigate PAK1 function in extravasation, $PAK1^{-/-}$ monocytes, identified by the monocyte surface marker CD14 (Souques et al., 1997), could be purified from the mouse peripheral blood (as described in (Allport et al., 2000)). A role for PAK1 in monocyte extravasation could be established through plating $PAK1^{-/-}$ monocytes onto a murine endothelial cell monolayer and to investigate monocyte transmigration (Allport et al., 2000). However, few monocytes can be harvested from a mouse making transmigration studies difficult. Another possibility is the use of intravital microscopy. This allows the *in vivo* observation of leukocytes rolling, adhering and transmigrating through the endothelium (Huang et al., 2006). Use of this technique in WT and $PAK1^{-/-}$ BMMs would help establish whether PAK1 is required for extravasation. However, in $PAK1^{-/-}$ mice, it is possible that endothelial responses could inhibit monocyte extravasation. Conditional knockout (Shen et al., 2005b; Wells et al., 2004) of PAK1 in endothelial and haematopoietic cells would

allow determination of whether PAK1 is required within leukocytes or the endothelium for extravasation. Alternatively, adoptive transfer could be utilised (Belanger and St-Pierre, 2005). Irradiated WT mice would be given a $PAK1^{-/-}$ bone marrow transfusion to establish WT mice producing $PAK1^{-/-}$ haematopoietic cells. Intravital microscopy could then be performed to observe $PAK1^{-/-}$ cell extravasation through a WT endothelium. The opposite experiment could also be performed to study WT cell extravasation through a $PAK1^{-/-}$ endothelium.

Another experiment which could provide evidence for a role of PAK1 in macrophage extravasation and migration *in vivo* would be injection of thioglycolate into the peritoneum (Potter et al., 2003). Performed in WT and $PAK1^{-/-}$ mice, the induction of peritonitis would allow evaluation of $PAK1^{-/-}$ BMMs ability to locate and target the site of the immune response highlighting any defects in $PAK1^{-/-}$ monocytes/BMMs. Intravital microscopy could also be used to observe macrophage targeting to the site of inflammation (La et al., 2003).

Once within the tissues, monocytes differentiate into macrophages which chemotax towards chemo-attractants such as C5a. Despite reports using both DNA constructs and RNAi indicating a role for PAK1 in cell migration (Adam et al., 1998; Kiosses et al., 1999; Zhou et al., 2003) and chemotaxis (Li et al., 2003b; Rousseau et al., 2006; Wang et al., 2002), there were no differences in persistence or migratory speed in $PAK1^{-/-}$ BMMs. (Chapter 5.1 and 5.2). The lack of contribution of PAK1 to cell migration was confirmed further through scratch assays, which indicated $PAK1^{-/-}$ BMMs were capable of polarising and migrating into the scratch (Chapter 5.3). Previous data reported PAK1 is required for lamellipodial polarisation in scratch assays (Cau and Hall, 2005), but this was not evident in the $PAK1^{-/-}$ BMMs. $PAK1^{-/-}$ BMMs also have no defects in CSF-1-induced chemotaxis (Chapter 5.4 and 5.5) suggesting PAK1 is not required for macrophage migration or chemotaxis towards CSF-1. However, PAK2, the other predominantly expressed isoform in BMMs (Chapter 3.1), was activated in macrophages undergoing chemotaxis towards RANTES (Weiss-Haljiti et al., 2004), raising

the possibility that PAK2 is involved in chemotaxis. The possibility also exists that the apparent lack of PAK1 function in migration is due to PAK family redundancy. To determine whether PAK2 is the key PAK isoform in BMM chemotaxis, inhibition of PAK2 and both PAKs could be performed. Although a PAK2-null mouse was embryonically lethal (Dr J. Chernoff – personal communication), use of PAK2-specific RNAi in WT and PAK1$^{-/-}$ BMMs would establish whether PAK2 was required for chemotaxis. RNAi has been performed in BMMs (Kim et al., 2005) although attempts to perform PAK RNAi transfection were unsuccessful. Use of alternative transfection reagents, however, may rectify this. The PAK AID could also be used to inhibit the group A PAKs present in BMMs, determining their role in macrophage migration. RNAi could also be utilised to study differences in BMMs behaviour with acute or chronic knockout of PAK1. Chronic disruption of PAK1 could allow the generation of compensatory pathways within the BMMs which were not identified here. Acute disruption with RNAi provides less opportunity for compensation and comparisons between the two techniques may identify evidence for compensation with the PAK1$^{-/-}$ BMMs.

A number of the reports that implicate PAK in chemotaxis utilise chemokines that stimulate GPCRs, whereas CSF-1 signals via an RTK. This difference could underlie the absence of a defect in CSF-1-stimulated PAK1$^{-/-}$ BMMs. To establish whether PAK1 is only involved in GPCR-mediated chemotaxis further studies should be performed. Chemokines such as CXCL1 were previously used to identify PAK1 as a regulator of chemotaxis (Wang et al., 2002). CXCL1 could be used with PAK1$^{-/-}$ BMM in Dunn chamber and Transwell filter assays to determine whether PAK1 was essential for chemokine-induced chemotaxis or whether PAK1 involvement is cell type specific. It would also be possible to study the role PAK1 has in T-cell and neutrophil function to investigate potential PAK1 cell-specific responses.

At present, no immune response defects have been reported for the PAK1$^{-/-}$ mouse. However, the PAKs have been implicated in phagocytosis. PAK1 (Dharmawardhane et al., 1999) and PAK2 (Robinson and Badwey, 2002) have been localised at sites of developing phagosomes in neutrophils. PAK1

was also localised at FcγR-mediated phagosomes in murine macrophages (Diakonova et al., 2002) and in U937 myeloid cells, where a potential link between Src, an Nck-PAK1 complex and regulation of NADPH oxidase respiratory burst was indicated (Izadi et al., 1998). Upstream of PAK1, both Rac1 and Cdc42 are known to regulate phagocytosis although both GTPases probably have different contributions. Rac1 activation of PAK1 and resultant JNK activity acts downstream of CD66-mediated phagocytosis of *Neisseria gonorrhrae* (Hauck et al., 1998), whilst Cdc42, RhoB, PAK1 and ROCK activation was required for mannose receptor-mediated phagocytosis of *Pneumocystis* organisms in alveolar macrophages (Zhang et al., 2005). PAK1 may be generally involved in the internalisation of extracellular molecules, since PAK1 contributes to macropinocytosis and pinocytic vesicle cycling in NIH3T3 fibroblasts (Dharmawardhane et al., 2000).

Following phagocytosis in leukocytes, pathogens within the phagosome are killed through the process of respiratory burst and the resultant influx of compensating ions (see review (Segal, 2005)). This involves the production of reactive oxygen species (ROS), including superoxide anion ($O_2^-$), hydroxyl radical ($OH^-$) and hydrogen peroxide ($H_2O_2$). Superoxide anion production occurs via the NADPH oxidase complex, an enzyme that is inactive in resting phagocytic cells and becomes activated through a number of mechanisms including binding of chemotactic peptides or opsonised particles. Upon stimulation, the various subunits of NADPH oxidase ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac) translocate to the cell membrane, bind flavocytochrome $b_{558}$ and become activated (see review (Groemping and Rittinger, 2005)). Rac1 and Rac2 were both identified as regulators of $p67^{phox}$ in neutrophils, whilst Cdc42 may act as a competitive inhibitor of Rac-induced NADPH activation (Diebold et al., 2004). PAK1 also regulates NADPH oxidase activation through phosphorylation of $p47^{phox}$ (Knaus et al., 1995; Martyn et al., 2005) whilst PAK2 has been identified as a kinase for the $p67^{phox}$ subunit (Ahmed et al., 1998). A potential Src-Cbl-Nck-PAK1 pathway is implicated in the activation of respiratory burst after FcγR-mediated phagocytosis (Izadi et al., 1998). Determination of a definitive role for PAK1 could be established through the use of $PAK1^{-/-}$ BMMs and their ability to kill bacteria as a

measure of NADPH oxidase activity. This could be done by incubation of BMMs with bacteria (e.g. *E. coli*) and subsequent lysis and plating of intracellular bacteria onto agar to determine the number of colonies formed (Laroux et al., 2005). This would indicate the efficiency at which the BMMs killed the bacteria after phagocytosis. Alternatively, the fluorogenic substrate lucigenin can be utilised to establish NADPH oxidase activity. When oxidised, lucigenin emits light which can be observed through a luminometer and quantitated to measure respiratory burst upon macrophage stimulation (Minkenberg and Ferber, 1984). These experiments should establish whether PAK1 is required for the respiratory burst in macrophages.

Use of the PAK1$^{-/-}$ BMMs would give a clear indication of whether PAK1 is essential for phagocytosis. Jersmann *et al.* developed a flow cytometry based phagocytosis assay (Jersmann et al., 2003) that was used in preliminary studies of WT and PAK1$^{-/-}$ BMMs phagocytosis of apoptotic and necrotic T cell populations. These indicated no differences between BMM genotypes, although further research is required to confirm this. This suggests that PAK1 is not essential for BMM phagocytosis of apoptotic or necrotic cells.

Another important function of the immune system is the release of pro-inflammatory cytokines which promote the attraction and activation of macrophages and other leukocytes at the site of inflammation. Macrophages secrete cytokines (Lucas et al., 2003) and PAK1 has been implicated in cytokine release through activation of the transcription factor NF-$\kappa$B upon *Helicobacter pylori* infection of epithelial cells (Foryst-Ludwig and Naumann, 2000). A likely mechanism for PAK1 induction of cytokine release is via the MAPK pathways. JNK and p38 signalling were both required for TNF$\alpha$ release after LPS stimulation (Ciallella et al., 2005; Shen et al., 2005a) whilst ERK1/2 was required for TNF$\alpha$ release in *Mycobacterium avium* infected macrophages (Lee and Schorey, 2005). The decrease in MAPK signalling observed in PAK1$^{-/-}$ BMMs suggests a possible defect in the MAPK-induced release of pro-inflammatory cytokines. CSF-1 stimulation was shown to induce TNF$\alpha$ transcription in WT BMMs (Chapter 3.4) suggesting that CSF-1

may induce cytokine stimulation via the MAPK pathway and via PAK1. Use of the PAK1$^{-/-}$ BMMs in Taqman experiments would determine whether PAK1 was involved in CSF-1-induced upregulation of cytokine expression.

The PAK1$^{-/-}$ BMMs could be used to determine a role for PAK1 in cytokine release. It would also be possible to differentiate the bone marrow cells into mast cells, specialised cytokine-secreting cells, using IL-3 and SCF (Ali et al., 2004). To investigate cytokine release in these cell types, it would be possible to use a multiplexed particle-based flow cytometric assay. Briefly, microspheres act as a solid support for an immunoassay which can be used on a flow cytometer. Several different sets of spheres are produced which are cytokine-specific and have distinct proportions of red and orange fluorescent dyes. This allows separation of bead sets and hence the detection of different cytokines within a single sample. Up to 15 separate cytokines can be detected from a single sample of tissue culture medium (Vignali, 2000). Use of this flow cytometry-based system should allow the identification of a number of cytokines and determine whether PAK1$^{-/-}$ BMMs and mast cells are defective in release of cytokines upon stimulation.

Interestingly, macrophages have been implicated in the progression of cancer. Macrophages induce tumour cell migration through formation of a CSF-1-EGF paracrine loop and in the processes of intravasation and angiogenesis (Chapter 1.4.1). It is possible that PAK1 may influence these macrophage functions in cancer and have a role in tumour progression. Although PAK1 was not required for macrophage migration or chemotaxis towards CSF-1 (Chapter 5.1 and 5.6), it may still be required for production or secretion of EGF. Mammary tumour cells stimulate macrophages through CSF-1 which in turn induce tumour cell migration through secretion of EGF (Wyckoff et al., 2004). The role of PAK1 in MAPK and NF-$\kappa$B signalling (Bagrodia et al., 1995; Beeser et al., 2005; Frost et al., 2000) suggest it may influence EGF production or possibly secretion. Use of Taqman quantitative RT-PCR would determine EGF mRNA levels and could be used to investigate whether PAK1 affects EGF transcription.

PAK1 could also potentially be involved in invasion and intravasation of metastatic cancer cells. Macrophages produce podosomes in a CSF-1 and PI3-K-dependent manner (Wheeler et al., 2006) and PAK1 has been implicated in this process (Webb et al., 2005). Macrophage podosomes are believed to degrade the extracellular matrix (ECM) (Yamaguchi et al., 2006) and so may remodel the matrix surrounding cancers, allowing migration and invasion. Alternatively, it was proposed that a similar CSF-1-EGF paracrine loop observed for promotion of migration also exists to promote cancer cell production of invadopodia, membrane protrusions involved in degrading and pushing into the extracellular matrix (Yamaguchi et al., 2006). Use of the PAK1$^{-/-}$ BMMs would allow investigations into whether podosome formation was inhibited upon CSF-1 stimulation and whether the ability of macrophages to degrade the ECM was affected. ECM degradation can be analysed using fluorescently-conjugated fibronectin and gelatin (Yamaguchi et al., 2006). Indeed, the process of invadopodium formation was shown to be N-WASp and Arp2/3-dependent indicating it to be Cdc42-dependent (Yamaguchi et al., 2005) and suggesting a potential role for PAK1. Use of PAK1 RNAi or the PAK AID in the metastatic MTLn3 cell line as used in Yamaguchi *et al.* would determine whether PAK affects cancer cell invadopodium formation.

Macrophages have also been implicated in promotion of angiogenesis *in vivo* (Bingle et al., 2006). The PAK AID inhibited angiogenesis in an *in vivo* chick chorioallantoic membrane assay (Kiosses et al., 2002). PAK was also implicated in $\alpha_v\beta_3$-mediated promotion of angiogenesis through phosphorylation of c-Raf, although it was not required for VEGF- or $\alpha_v\beta_5$-mediated promotion (Hood et al., 2003). However, PAK1 may regulate VEGF production and/or secretion (Bagheri-Yarmand et al., 2000), suggesting a further level of PAK1-mediated regulation of angiogenesis. To investigate a function for PAK1 in macrophage-induced angiogenesis, T47D cell spheroids infiltrated with WT and PAK1$^{-/-}$ BMMs before implantation into nude mice could be used (Bingle et al., 2006). A role for PAK1 in macrophage function would be visible through changes to the level of angiogenesis.

These data highlight a number of potential functions for PAK1 in macrophages, from influencing phagocytosis and the oxidative burst to the induction of angiogenesis and extravasation. Further investigation into the role of PAK1 in normal macrophage function and in macrophage-mediated cancer progression is warranted.

## 7.2: PAK1 signalling in macrophage functions

$PAK1^{-/-}$ BMMs were identified as having a defect in lamellipodial stability during spreading (Chapter 6.4). This suggests PAK1 has a role in regulating membrane stability and so may affect other macrophage functions which rely upon controlled membrane dynamics, such as phagocytosis, cytokine release and extravasation. The PAK1 signalling pathways studied here may indicate the underlying causes of the membrane defects observed in $PAK1^{-/-}$ BMMs.

PAK1 signalling has been implicated in regulation of the cytoskeleton which may influence membrane dynamics. PAK1 regulates the early CSF-1-induced phosphorylation of LIMK in BMMs (Chapter 4.6) which would inhibit cofilin activity and, therefore, increase actin stability. LIMK regulation of cofilin and actin dynamics has previously been implicated in regulation of phagocytosis in U937 macrophage-like cells (Matsui et al., 2002) and in *Listeria*-induced phagocytosis and infection of non-phagocytic REF52 cells (Bierne et al., 2001). This is likely to be through regulation of actin dynamics at the cell membrane during formation of the phagocytic cup. Interestingly, tight regulation of LIMK activity appears to be critical for phagocytosis as both over-expression and inhibition of LIMK activity inhibits phagocytosis (Bierne et al., 2001). This indicates that $PAK1^{-/-}$ BMMs may have a defect in phagocytosis because of reduced LIMK activity.

Other PAK-mediated signalling pathways could also affect phagocytosis. The MAPK pathways (Chapter 4.3) and MLC (Chapter 4.7) have both been implicated in phagocytosis, and the MAPK pathways may affect contractility through regulation of MLCK and MLC phosphorylation (Araki et al., 2003;

Mansfield et al., 2000; Olazabal et al., 2002). Contraction is essential for closure of the phagosome (Reville et al., 2006) and is therefore, a further pathway whereby PAK1 may influence phagocytosis.

These pathways regulate the underlying cytoskeletal dynamics during phagocytosis. However, PAK1 regulation of the cytoskeleton may also influence membrane ruffling, a process essential for particle engulfment (Coelho Neto et al., 2005). CSF-1-stimulated PAK1$^{-/-}$ BMMs had reduced membrane ruffling (Chapter 4.10) and the possibility of a phagocytosis defect in these macrophages due to defective ruffling deserves investigation. However, the ruffling required for phagocytosis is likely to be localised and a reduction in overall membrane ruffling is insufficient to indicate a defect in phagocytosis. To investigate BMMs signalling activity during phagocytosis, IgG opsonised beads could be utilised to stimulate phagocytosis without introducing a different cell type to the BMMs. This should allow identification of pathways that are activated during phagocytosis in BMMs and determine the impact of loss of PAK1 upon these pathways.

PAK1 regulation of membrane dynamics may also influence the release of cytokines. PAK1 was implicated downstream of Rac1-induced exocytosis (Li et al., 2003a) whilst myosin II, which is regulated through the PAK1 targets MLCK and MLCP, is involved in tear protein exocytosis (Jerdeva et al., 2005) and in vesicle transport and fusion (Neco et al., 2004). The PAK partner $\beta$-PIX is also involved in neuronal exocytosis (Audebert et al., 2004; Chahdi et al., 2004). Use of the multiplexed particle based flow cytometric assay and Taqman RT-PCR assay, as described above, would establish whether PAK1 is required for cytokine expression or release in BMMs.

Although PAK1 was not essential for BMM migration or chemotaxis, it was required for spreading and adhesion. PAK1 regulation of LIMK and MLC phosphorylation could influence lamellipodial stability as could PAK1 regulation of ERK phosphorylation at the leading edge. Disruption of these signalling processes through loss of PAK1 may inhibit the ability of monocytes/leukocytes to extend processes through the endothelial

monolayer. Since PAK1 affects adhesion (Chapter 1.3.5 and Chapter 6.17), $PAK1^{-/-}$ monocyte adhesion to the endothelium may also be defective. Analysis of PAK-mediated signalling pathways and adhesion formation through immunofluorescence of $PAK1^{-/-}$ monocytes/leukocytes undergoing transmigration could identify any changes in protein localisation and activation. However, in the process of extravasation, signalling within the endothelium is also important. MLC activity in endothelial cells has been implicated in extravasation (van Hinsbergh and van Nieuw Amerongen, 2002) and so PAK1 is also likely to be critical in the endothelium. Therefore, *in vivo* assays of macrophage targeting to the peritoneum must also consider the possibility that $PAK1^{-/-}$ monocytes cannot transmigrate due to defective endothelial signalling rather than monocyte signalling. Use of $PAK1^{-/-}$ endothelial cells or PAK1 RNAi on an endothelial cell line such as human umbilical vein endothelial cells (HUVECs) could establish defects in endothelial cell signalling either upon cytokine stimulation or under shear stress (Wojciak-Stothard and Ridley, 2003).

Overall, data presented in this thesis support a role of PAK1 in cell spreading and in signalling-mediated regulation of the cytoskeletal network. However, it questions the involvement of PAK1 in cell migration and chemotaxis, at least in BMMs, suggesting that PAK1 is not essential and that further experimentation is required to determine whether the PAK family affect this process.

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